Polyamine conjugates with potential as therapeutic targets

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POLYAMINE CONJUGATES WITH POTENTIAL AS THERAPEUTIC TARGETS

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Abstract

In this work conjugates of polyamines have been designed and synthesised for therapeutic evaluation against cancer cells and trypanosomal targets.

The positive charges carried by endogenous polyamines at physiological pH give them high affinity for DNA leading to groove-binding. A dedicated cellular polyamine uptake system has been characterised which is upregulated in rapidly proliferating cells (e.g. many cancers). Anthracene and acridine are cytotoxic DNA intercalators. This cytotoxicity was enhanced by the design and synthesis of conjugates with polyamines to give stronger DNA binding through bifunctional modes of DNA interaction (simultaneous intercalation and groove binding), and potential cellular uptake by the polyamine transporter.

Regioselective protection of the tetraamine spermine allowed nucleophilic conjugation to the 9- position of the polyaromatic, either through an amide (anthracene and acridine) or by direct substitution of a phenoxide (acridine), by a primary amine. Acridine conjugates were also synthesised with a variety of spacers between the acridine and the spermine moieties. These conjugates were used in cytotoxicity assays by M. A. Qarawi, University of Bath, *in vitro* against B16 murine melanoma cells. The most cytotoxic conjugate was a 9-aminoacridine with a 5-aminopentanoic acid derived spacer with a cytotoxic EC_{50} of 2.7×10^{-7} M.

Further conjugates were prepared replacing spermine with tetraamines with different configurations of methylene groups. These compounds were produced by solid phase organic synthetic techniques and employed the 4-pentenoyl protecting group for the first time in solid phase polyamine synthesis.

Syntheses were designed for 23- 24- and 28-membered macrocyclic analogues of cadabicine, a spermidine containing plant natural product. Polyamine protection controlled the polyamino regiochemistry and macrocyclisation was achieved by forming diphenyl ether bonds through S_NAr displacement of an *ortho*-nitro activated fluorine by a phenol. These conjugates will be tested as inhibitors of trypanothione reductase, a key medicinal target against trypanosomal parasites.

Acknowledgements

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Finally I would like to thank my family and Sue for their continuing support.

Abbreviations:

AdoMet	*S*-adenosylmethionine
AdoMetDC	*S*-adenosylmethionine decarboxylase
anhyd.	anhydrous
BAP	borane-pyridine complex
BCBD	N^1,N^4-bis(7-chloroquin-4-yl)butane-1,4-diamine
BOC	*t*-butoxycarbonyl
DCC	dicyclohexylcarbodiimide
DCU	dicylcohexylurea
DEHSPM	diethylhomospermine
DENSPM	diethylnorspermine
DESPM	diethylspermine
DFMO	difluoromethylornithine
DMAP	*N*,*N*-dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DOGS	dioctadecylamidoglycylspermine
DPPES	dipalmitoylphosphatidylethanolamine
equiv.	equivalents
EtOH	ethanol
FTIR	fourier transform infra-red spectroscopy
HOBt	*N*-hydroxybenzotriazole
RP-HPLC	reverse phase high performance liquid chromatography
IR	infra-red
MeOH	methanol

MGBG	methylglyoxal-bis(guanohydrazone)
MP	melting point
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	nuclear magnetic resonance
ODC	L-ornithine decarboxylase
PAO	polyamineoxidase
ppm	parts per million
SPOS	solid phase organic syntheis
SSAT	spermidine/spermine-N^1-acetyltransferase
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TMSI	trimethylsilyliodide
TR	trypanothione reductase
UV	ultra-violet
VSCC	voltage sensitive calcium channels
Z	benzyloxycarbonyl

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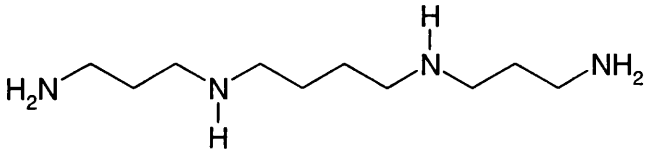
Chapter 1

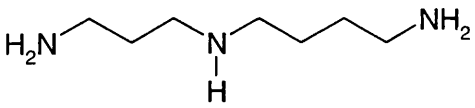
Introduction

1.1 General Introduction to Polyamines

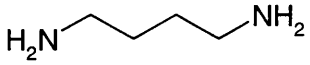
Polyamines are structurally simple low molecular weight aliphatic amines. They are cellular components, essential for normal growth and replication (Tabor and Tabor, 1964). The two key endogenous polyamines are the tetraamine spermine **1** (bis-N^1,N^2-(3-aminopropyl)-1,4-diaminobutane; 4,9-diaza-1,12-diaminododecane) and the triamine spermidine **2** (*N*-(3-aminopropyl)-1,4-diaminobutane; 4-aza-1,8-diaminooctane). The closely related diamine putrescine **3** (1,4-diaminobutane) is also of importance both as the biosynthetic precursor of spermine **1** and spermidine **2** and due to its own (polyamine-like) biological activity.



Spermine **1**

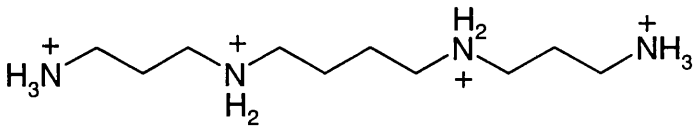


Spermidine **2**

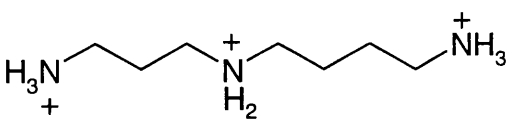


Putrescine **3**

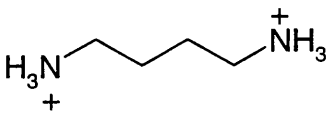
These compounds are essentially fully protonated at physiological pH (7.4) (Table 1.1) and it is the string of positive charges along their length which lies behind much of their biological activity, especially their high affinity at DNA and certain proteinaceous receptors.



Spermine **1**



Spermidine **2**



Putrescine **3**

Table1.1 Measured p*K*a values for simple amines

Amine	**p*K*a's**
Putrescine **3**	10.80, 9.63[a]
Spermidine **2**	11.56, 10.80, 9.52[b]
Spermine **1**	11.50, 10.95, 9.79, 8.90[b]
Propylamine	10.54[a]
Butylamine	10.60[a]

(a. Albert and Serjeant, 1984; b. Takeda *et al.*, 1983)

Nature has evolved a system of biosynthetic and interconversion enzymes which keep intracellular polyamine concentrations constant, normally in the μM range (Tabor and Tabor, 1984). At times of rapid cellular growth and division, the concentrations of endogenous polyamines can rise to mM levels, a situation particularly apparent in cancer cells (Tabor and Tabor, 1984). There also exists a polyamine transport mechanism (Felschow *et al.*, 1995) which allows cells to scavenge exogenous polyamines from their environment which they can substitute for those created by *de novo* synthesis, a system which may also be reversed to allow cells to excrete excesses (Grillo and Colombatto, 1994). Their crucial cellular roles and specific enzymatic and transport pathways make polyamines desirable targets for therapeutic intervention. The plethora of polyamine conjugates found throughout the plant and animal kingdoms means there are many natural products which can be assessed for therapeutic activity through interaction with macromolecular biological anions (e.g. proteins, DNA), as inhibitors of enzymes with polyamine or polyamine derived substrates or through interference with intracellular polyamine levels.

The importance of the specific polyamine structures to cellular processes becomes apparent when the amount of energy expended in their biosynthesis is considered. If they simply fill a role of charge neutralisation, then metallic cations would be more energy efficient replacements. If the spacing between the positive charges is also of importance, then potentially a polylysine could substitute. Research is still ongoing and the knowledge of the specific mechanisms of polyamine action is continually being updated. Due to their physiological activities, both endogenous and synthetic polyamines as well as polyamine containing conjugates are of considerable interest in the field of medicinal chemistry. Much work has been published on polyamine based therapies against cancers and against protozoal diseases. Small molecule polyamine conjugates from certain spiders and wasps have been shown to block cation channels, leading to interest in them both as pharmacological tools and as treatments for neurodegeneration.

Polyamines form the backbone for iron scavenging siderophores used by microbes to sequester the iron they require from their environment. These compounds and their analogues are of interest to investigate iron transport in cells and the role of iron in infection as well as treatments for diseases associated with iron imbalance. Triamines have been used in conjugates to effect site-selective DNA and RNA cleavage, the acid-base cooperativity between adjacent amine groups being the key to their activity. The affinity of polyamines for DNA has been the basis for a number of synthetic conjugates which condense and deliver DNA particles to cells in order to express genes. This is a key strategy in gene therapies which could eventually treat genetically caused illnesses such as cystic fibrosis. For reviews of medicinal chemistry research in these areas, see: Marton and Pegg, 1995 and Blagbrough *et al.*, 1997.

1.2 Polyamine Biosynthesis

The polyamine biosynthetic pathway has been characterised by a number of key enzymes which synthesise or metabolise polyamines, maintaining their required intracellular concentrations (Fig. 1.1) (Marton and Pegg, 1995). In mammals, synthesis begins from the α-amino acid L-ornithine, which itself is derived from DNA-encoded L-arginine. L-Ornithine decarboxylase (ODC) performs decarboxylation to yield the diamine putrescine **3**. *S*-Adenosylmethionine (AdoMet) is decarboxylated by *S*-adenosylmethionine decarboxylase (AdoMetDC) to give a species (dcAdoMet) which is an aminopropyl donor. In conjunction with spermidine synthase and then spermine synthase, putrescine **3** is successively aminopropylated to give spermidine **2** and then spermine **1**.

In the metabolic pathway there are two key enzymes. Spermidine/spermine-N^1-acetyltransferase (SSAT) acetylates a propylamine moiety in either spermine or spermidine to give a substrate for polyamine oxidase (PAO) which then cleaves the acetylated propylamine. Both putrescine **3** and N^1-acetylspermidine can be readily excreted from the cell and putrescine **3** can also be degraded by diamine oxidase. Inhibitors have been found for all of the enzymes in both the catabolic and metabolic pathways and have been used in therapeutic regimes (Marton and Pegg, 1995). The polyamine biosynthetic pathway is strongly repressed in the presence of polyamines so, when depletion begins to occur, the catabolic enzymes make large compensatory increases in their activity. There is a rapid cycling of the enzymes, ODC has a half-life of 20 minutes, so often inhibitors have to be administered for extended periods of time for continued effectiveness. Overall these inhibitors are cytostatic rather than cytotoxic making polyamine inhibitors more useful as compounds to be co-administered alongside drugs with alternative mechanisms of action rather than as therapies in their own rights.

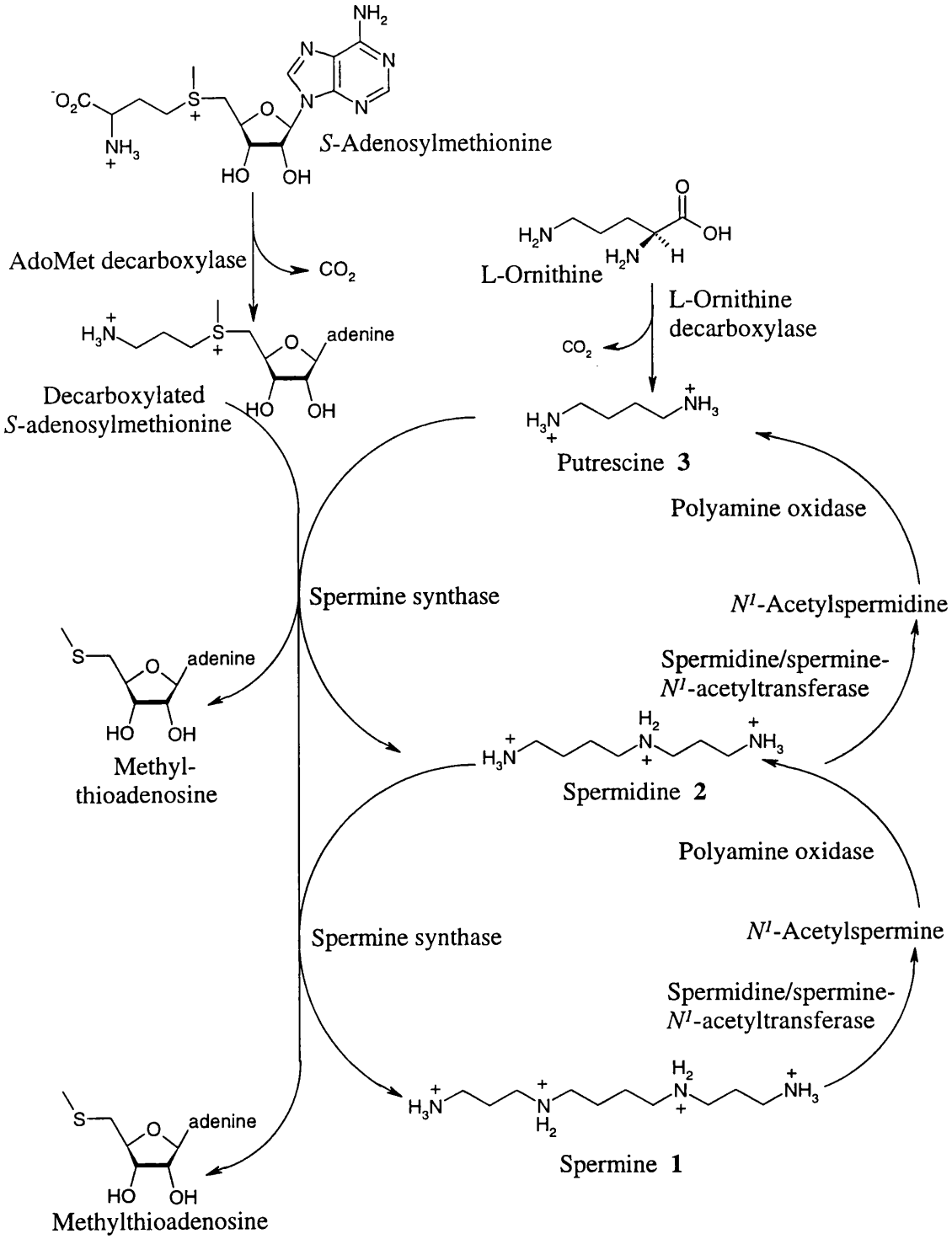
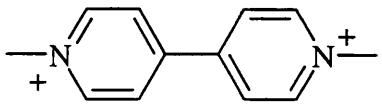


Fig. 1.1

(after Marton and Pegg, 1995)

1.3 Polyamine Transport

Many cell types have been shown to possess dedicated polyamine uptake mechanisms which are distinctly separate to amino-acid transport systems (Morgan, 1990; Seiler and Dezeure, 1990; Grillo and Colombatto, 1994). The polyamine transporter is upregulated under circumstances where cells require greater polyamine quantities than can be produced by *de novo* synthesis. It can be induced by endogenous hormones and growth factors as well as by depleting the cell of polyamines by biosynthetic enzyme inhibitors. The transporter is upregulated in cancer cells and in cells which have been stimulated to proliferate (Seiler *et al.*, 1990) and it is regulated by the concentrations of intracellular polyamines. It has been shown that induction of the system actually involves synthesis of new transporter protein rather than modifying existing protein in a way which modulates polyamine affinity (Mitchell *et al.*, 1992). Excess of intracellular spermine **1** or spermidine **2** is considered to be the signal to terminate the uptake by causing the production of a short-lived regulatory protein (Mitchell *et al.*, 1992). Investigations have been carried out to investigate the variability in structure of synthetic polyamines which are recognised and transported (Porter *et al.*, 1984; Felschow *et al.*, 1995 and 1997). This work is of particular importance for the design of polyamine derived anti-cancer agents which can be designed to use this system (Cohen and Smith, 1990; Marton and Pegg, 1995). Also, in related studies, the toxicity of the dipyridinium species paraquat (*N,N'*-dimethyl-4,4'-dipyridinium) **4** has been shown to be via a specific transporter (Smith *et al.*, 1982; Brooke-Taylor *et al.*, 1983). This gives a basis for the lung specific toxicity observed in paraquat **4** poisoning.

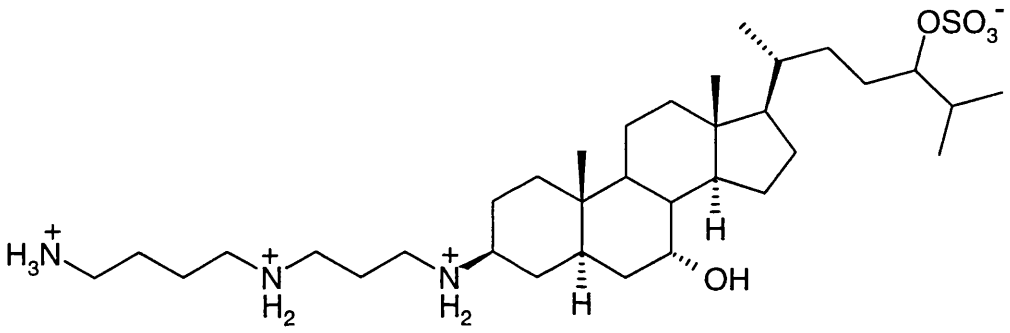


Paraquat **4**

1.4 Novel Polyamine Natural Products

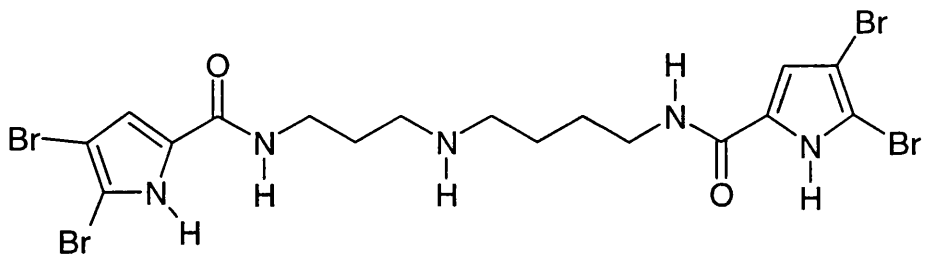
Polyamines have been isolated from many natural sources. There are an especially large number of plant alkaloids containing polyamine moieties (Guggisberg and Hesse, 1983 and 1998). Many of these natural products are of interest to medicinal chemists, providing leads in the development of novel therapeutic agents, illustrated below by four examples of polyamines isolated from plants or cultures.

The sterol sulfate-spermidine conjugate squalamine **5** (Moore *et al.*, 1993), isolated from the liver and gallbladder tissues of the dogfish shark *Squalus acanthias*, shows antitumour, antimicrobial and fungicidal activities. It also induces osmotic lysis of protozoa (Moriarty *et al.*, 1994 and 1995; Sadownik *et al.*, 1995).



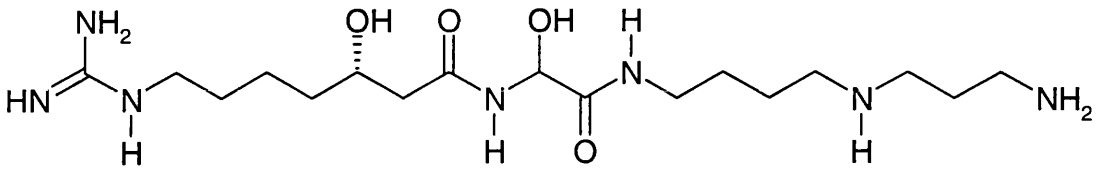
Squalamine **5**

Pseudoceratidine **6**, is a terminally diacylated spermidine derivative which has been isolated by the research group of Tsukamoto from the marine sponge *Pseudoceratina purpurea* (Tsukamoto *et al.*, 1996). It has potential as an anti-fouling agent in the paint applied to ship hulls to inhibit the undesirable growth of both macroscopic and microscopic organisms (Ponasik *et al.*, 1996 and 1998).



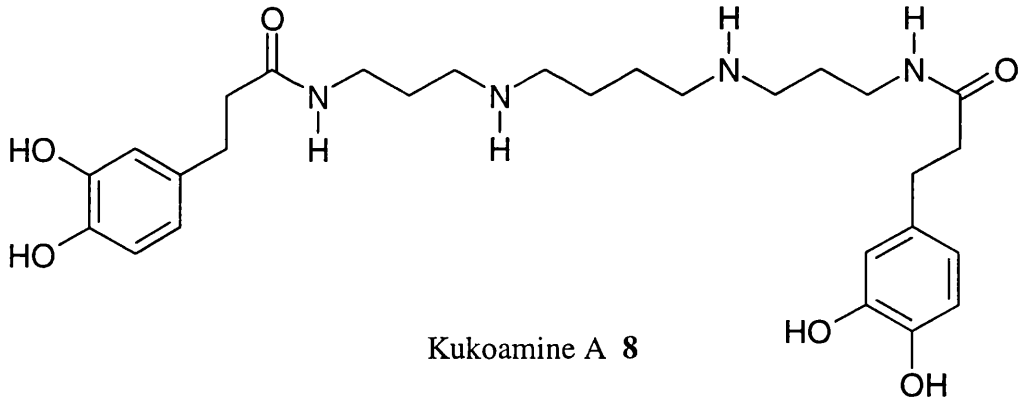
Pseudoceratidine **6**

Spergualin **7** is a peptide conjugate of spermidine **2** isolated from culture filtrates of *Bacillus laterosporus* (Nishizawa *et al.*, 1988). It shows antibiotic and antitumour activity and minor modifications to the structure yield potent immunosupressive compounds with effectiveness in preventing organ rejection after transplant (Umeda *et al.*, 1987; Nishizawa *et al.*, 1988).



Spergualin **7**

Kukoamine A **8** is a spermine conjugate terminally di-acylated with dihydrocaffeic acid isolated from *Lycium chinese* (Chantrapromma and Ganem, 1981). It has been shown to have antihypertensive activity (Funayama *et al.*, 1980) and more recently to be a potent, selective inhibitor of trypanothione reductase *vide infra*, a key target in current research against trypanosomal parasites (Ponasik *et al.*, 1995).

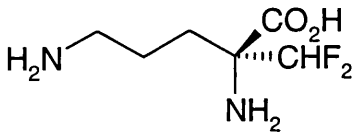


Kukoamine A **8**

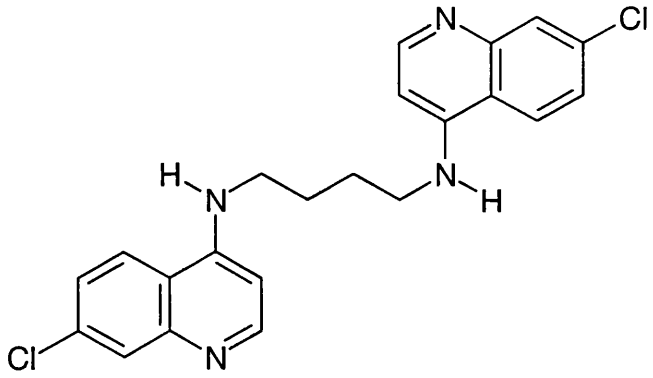
1.5 Polyamine Antiparasitic Conjugates

Parasitic disease continues to be a major problem for world-wide health. Malaria threatens half of the global population and the incidence of strains of protozoa resistant to the current drugs is ever increasing (Marton and Pegg, 1995). The acquisition and the use of polyamines are sufficiently different in such parasites compared to in mammals

that therapies based on polyamine mechanisms are attractive. DFMO **9** is an irreversible inhibitor of ODC, the enzyme which generates putrescine **3** from L-ornithine at the beginning of polyamine biosynthesis. Malaria causing parasites of the genus *Plasmodium* develop in mammalian erythrocytes. In this environment, there are low levels of polyamines which the parasite can easily sequester, so it must synthesise its own to provide enough to facilitate the synthesis of the proteins required for its growth and development (Assaraf *et al.*, 1984; Whaun and Brown, 1985). DMFO **9** inhibition of putrescine **3**, and hence polyamine biosynthesis in *in vitro* models leads to the arrest of parasite development at an early stage (Assaraf *et al.*, 1984). Exposure to putrescine **3** leads to a reversal of the effects, spermidine levels rise and development resumes. Unfortunately, *in vivo* DMFO **9** proves to be less effective. It has been suggested that parasites can take up putrescine **3** from plasma by increasing transport across the cell membrane of the infected erythrocytes (Singh *et al.*, 1997). In order to counter this, investigation has recently been started into combination therapy with N^1, N^4-bis(7-chloroquinolin-4-yl)butane-1,4-diamine (BCBD) **10**, an inhibitor of the putrescine uptake mechanism (Singh *et al.*, 1997).

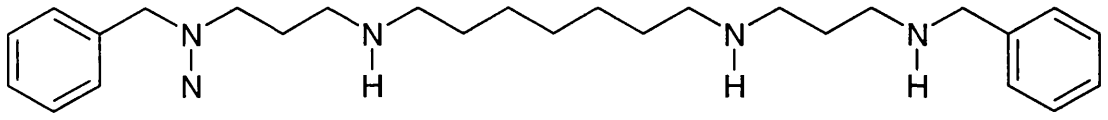


DFMO **9**

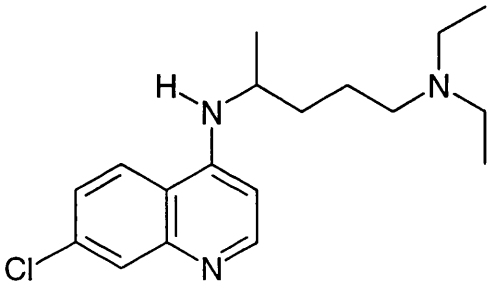


BCBD **10**

Another compound which shows good activity against *P. falciparium in vitro* and cures *P. bergi* infections in mice is the terminally di-benzylated tetraamine **11** (Bitonti *et al.*, 1989). Its development came about through structure-activity studies sparked by the success shown by dialkylated tetraamines against cancer cell lines (Edwards *et al.*, 1991) *vide infra.* Polyamine **11** is used in conjunction with DFMO **9** which stimulates its uptake by the parasite. As with the anti-cancer compounds, once in the cell it is thought to displace natural polyamines from critical binding sites disrupting normal cellular processes. Although less active than chloroquine **12**, this treatment is more potent than other therapeutic agents used to treat chloroquine resistant malaria.



11

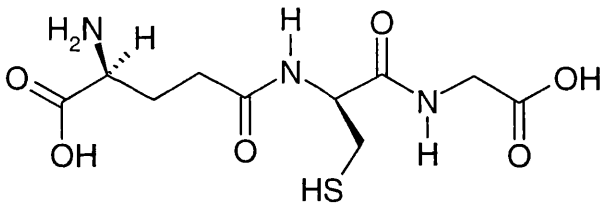


Chloroquine **12**

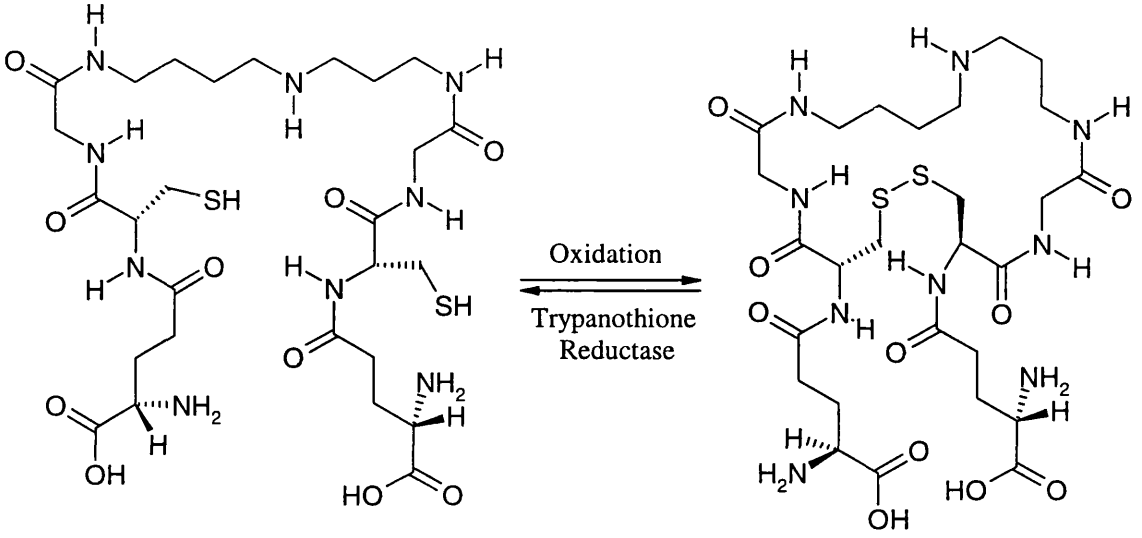
Other genera of parasites causing human disease are *Trypanosoma* and *Leishmania. T. brucei gambiense* causes sleeping sickness, *T. cruzi* causes South American Chaggas disease and *Leishmania* species cause Leishmaniasis. DMFO **9** has been used to treat *T. brucei* infections (Marton and Pegg, 1995). The parasite's ODC enzyme turns over less rapidly than that of the mammalian host, which is desirable, but it is less sensitive to DFMO **9**. Despite this, DFMO has proved to be effective and in fact is the only new drug to be licensed to treat sleeping sickness in the last 40 years

(Marton and Pegg, 1995). The reason for its success probably lies with the differences in the way the parasite biosynthesises and uses polyamines compared to its host. At the simplest level, DFMO **9** will halt trypanosomal development through polyamine deprivation leaving it susceptible to attack by the host's immune system. Two other contributory factors might be the build up of massive concentrations of AdoMet and the cessation of the synthesis of trypanothione **14**.

In the parasitic system, AdoMetDC is not regulated in the same way as in mammalian systems, so that, when polyamine biosynthesis ceases, AdoMet continues to build up and is not metabolised (Byers *et al.*, 1991). Glutathione **13** is the tripeptide γGluCysGly which is present at mM concentrations to protect mammalian cells from toxic free-radicals and electrophiles, including those generated *in vivo* during xenobiotic metabolism. Trypanothione **14** is the parasitic equivalent of mammalian glutathione **13** (Fairlamb and Cerami, 1992). Trypanothione is a *bis*-glutathione conjugate with spermidine which cannot be produced if polyamine synthesis is halted. It has a dithiol **14** which is oxidised to a disulfide bridge **15** in the presence of active oxygen species. In order to regenerate trypanothione **14** the enzyme trypanothione reductase (TR) is used to cleave the disulfide bond. It has been shown that glutathione disulfide (the mammalian equivalent of trypanothione disulfide **15**) is not processed by trypanothione reductase and equally it has been proven that trypanothione disulfide is not processed by mammalian glutathione reductase (Krauth-Siegel *et al.*, 1987). This has lead to the belief that enzyme inhibitors can be designed which inhibit TR but do not affect glutathione reductase. Inhibition of TR would severely compromise the parasitic defence system against free-radicals, either naturally occurring or introduced by a drug (Chan et al., 1998).



Glutathione **13**

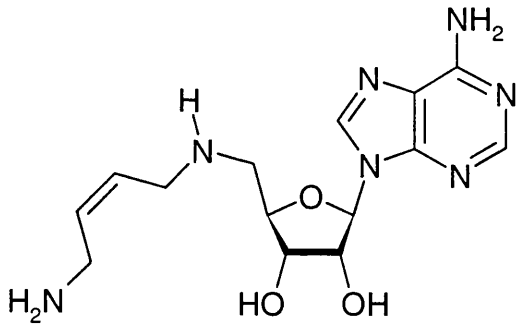


Trypanothione **14**

Trypanothione disulfide **15**

One method of anti-trypanosomal treatment is the administration of 5-nitrofurans such as nifurtimox (Walsh *et al.*, 1991) which undergo redox cycling with NAD(P)H generating more oxygen radicals than the trypanothione system can cope with.

For treatment with DFMO **9** to be successful the patient must be hospitalised for 14 days with intravenous DFMO for 6 h each day (Marton and Pegg, 1995). Fortunately, DFMO has low toxicity so there are few toxic effects from such long exposure, but it is expensive to produce, a major problem to the impoverished African nations where sleeping sickness is endemic. A more effective inhibitor of polyamine synthesis is AbeAdo **16** which inhibits the AdoMet enzyme. The most dramatic change shown in trypanosomes treated with AbeAdo is a greatly elevated concentration of AdoMet which is thought to be the cause of the antitrypanosomal effect (Byers *et al.*, 1991).



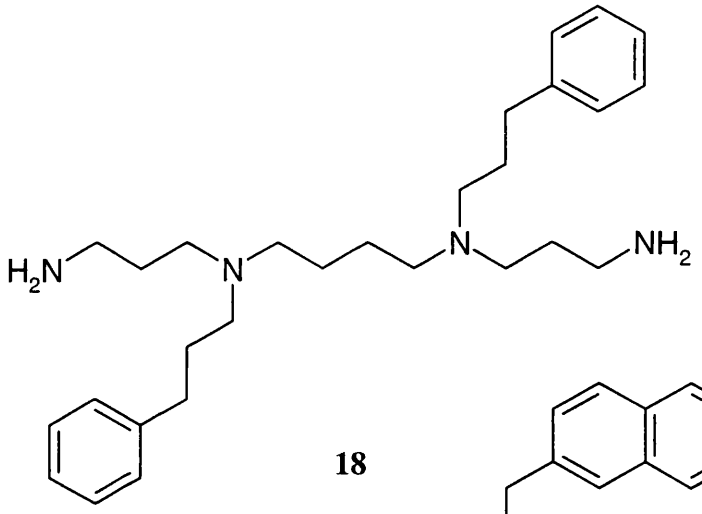
AbeAdo **16**

An important organism where DFMO **9** is not an effective treatment is *T. cruzi*. Investigations of this trypanosome have shown that during certain stages of its life-cycle it does not produce putrescine from either ornithine, as in mammals, or from arginine, a process common in most species except mammals and yeast (Tabor and Tabor, 1984). Instead, it synthesises polyamines from putrescine **3** and cadaverine (1,5-diamino-pentane) **17** which it scavenges from its host organism. Spermidine and spermine are synthesised as well as the equivalent tri- and tetraamines from cadaverine **17**.

Cadaverine **17**

As DFMO **9** has little effect, enzyme inhibitors for trypanothione reductase (TR) have become target combative agents. It has been known from the beginning of the twentieth century that trivalent aromatic arsenicals can be used to treat parasitic infections (Walsh *et al.*, 1991). Even after 80 years of use, the mechanism of action has only just been elucidated. The arsenic binds to trypanothione linking the thiols to form a macrocycle which is then an inhibitor of TR (Walsh *et al.*, 1991). The problem with the arsenical and nitrofuran drugs is their human toxicity so a specific inhibitor for TR would be anticipated to be much more acceptable, having little or no affinity for the mammalian glutathione reductase. TR inhibitors have been described, mostly taking the form of terminally substituted spermine and spermidine derivatives. The initial lead

was the plant natural product kukoamine A, N^1,N^8-bis(dihydrocaffeoyl)spermine **8** (Ponasik *et al.*, 1995). Other potent inhibitors include *N*-alkylated tetraamines diphenyl **18** and dinaphthyl **19**, and the *N*-alkylated dinaphthyl triamine **20** (O'Sullivan *et al.*, 1997).



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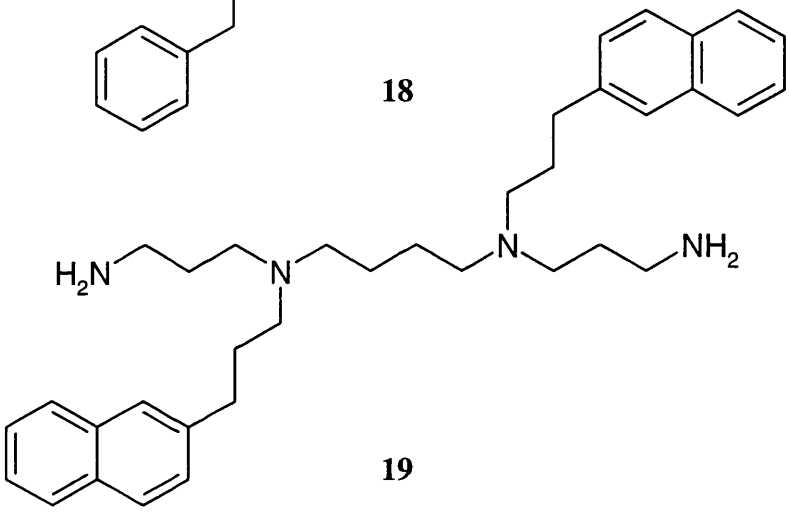

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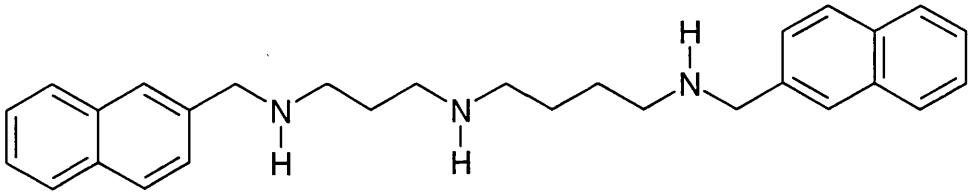


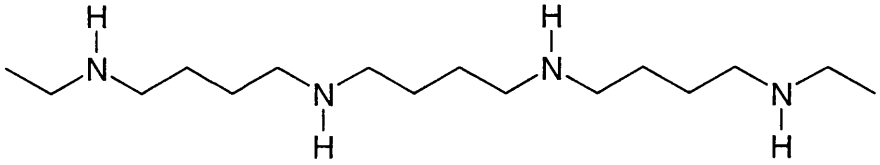
20

So far these compounds have only been shown to have trypanocidal activity *in vitro* and have failed to demonstrate any *in vivo* activity. They are still being investigated and have the potential to yield compounds with good activities and low host toxicities.

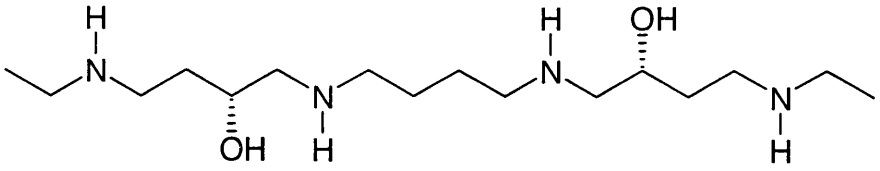
1.6 Polyamine Conjugates in the Treatment of Diarrhoea

In 1964, Tabor and Tabor reported that relatively high concentrations of spermine **1** and spermidine **2** are found in the gastrointestinal tract (Tabor and Tabor, 1964). Acting on this information, Tansy and co-workers were keen to characterise the physiological activity of polyamines in the gut, so initial investigations were carried out with polymers derived from ethylenimine, commonly available polyamine containing compounds with a variety of industrial uses (Melamed *et al.*, 1977). Branched-chain polymers were shown to inhibit gastric emptying in rats whilst linear structures had negligible physiological activity. From these results, it was thought that polyamines might find use as appetite suppressants or in prolonging the action of orally administered drugs. However, further experimentation employing oral administration to dogs showed a severe retching response (Tansy *et al.*, 1977). Investigations were also carried out with small molecule polyamines and synthetic close analogues (Belair *et al.*, 1981). Amongst these compounds, the naturally occurring polyamines were found to have a profound effect on gastric emptying whilst synthetic analogues were shown to have significantly lower activities.

In the course of HIV related infections, there is the frequent occurrence of serious diarrhoea either as a result of infection or as a side effect from certain drugs. So far this diarrhoea has been difficult to treat, existing drugs giving only partial responses and the therapy is accompanied by a high relapse rate. A new approach to treatment has been the use of synthetic polyamines, developed by Bergeron and co-workers (Bergeron *et al.*, 1996), to slow gut motility. After initial structure-activity assessment, DEHSPM was found to show anti-diarrhoeal activity in a castor-oil induced diarrhoea model in rats and has now been used to treat patients in the clinic. The drawback with this compound is chronic toxicity which is associated with the accumulation of metabolites.



DEHSPM **21**



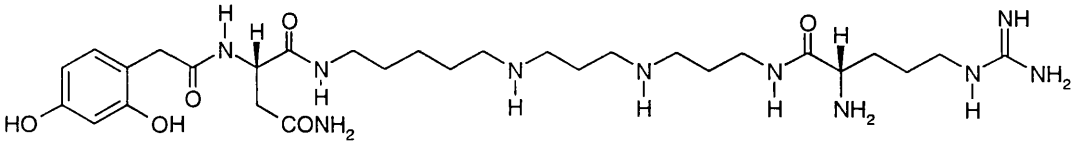
R, *R*-dioltetraamine **22**

In vivo studies have shown that DEHSPM **21** is first metabolised by *N*-de-ethylation. Normally, the next stage in polyamine metabolism would be removal of the 3-aminopropyl moieties through the action of SSAT and PAO. However, the remaining homospermine contains only 4-aminobutyl fragments and so is not metabolised, this leads to accumulation in the patient's tissues. This problem has been resolved by synthesising compounds substituted with hydroxyl groups (on tetrahedral carbon atoms of *R*-configuration) on methylenes located γ-to the ethylated amines **22**. These alcohol functional groups offer potential sites for enzymatic conjugation or oxidation leading to further metabolism and elimination. Studies showed that dioltetraamine **22** retains its gastrointestinal activity and has significantly reduced chronic toxicity, although the exact mechanism of degradation is not known (Bergeron *et al.*, 1996). These results illustrate the way in which toxicity associated with polyamines can be reduced without the loss of therapeutic effects.

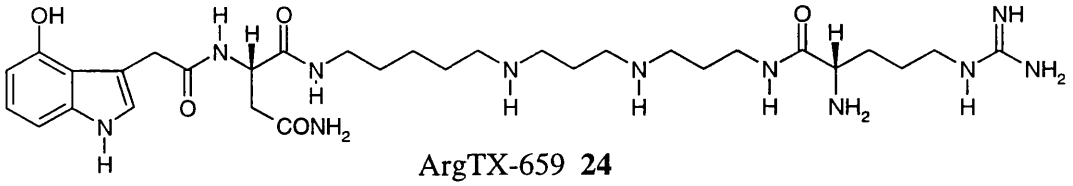
1.7 Polyamine Ion Channel Blockers

Spermine **1** is found in the venoms of certain spiders where it accompanies a range of small molecule phenols (Fischer and Bohn, 1957; Gilbo and Coles, 1964). From these venoms, many structurally novel polyamine amides **23-28** have now been isolated (for recent reviews of these argiotoxins, see: Blagbrough and Usherwood, 1992; Schäfer, *et al.*, 1994) and characterised pharmacologically (for reviews, see: Usherwood and Blagbrough, 1991; Carter, 1995; Mueller *et al.*, 1995). These low molecular weight polyamine containing toxins (argiotoxins) are potent and selective non-competitive antagonists of glutamate receptors, blocking the cation-selective channels associated with this excitatory α-amino acid (both NMDA- and non-NMDA glutamate receptors). Therefore, they have potential as pharmacological probes and as lead compounds for the design of drugs to treat neurodegeneration, especially stroke (Parks *et al.*, 1991; Blagbrough and Usherwood, 1992; Carter, 1995). However, unsymmetrical polyamine amides require total syntheses for sufficient material to be available for detailed pharmacological characterisation as well as for complete confidence in the structural assignments.

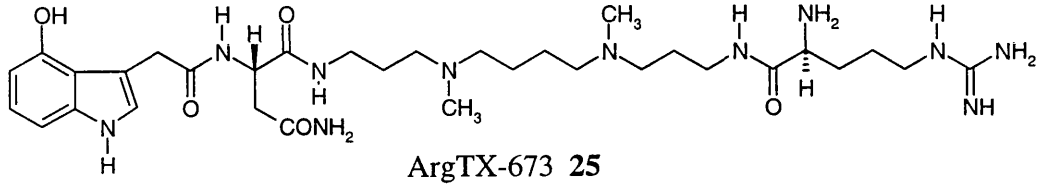
The novel polyamine FTX-3.3 **29** (Blagbrough and Moya, 1994) and the polyamine amide sFTX-3.3 **30** (Moya and Blagbrough, 1994) are important pharmacological tools for modulation of voltage-sensitive calcium channels (VSCC). These polyamines block VSCC with differential inhibition in mature rat cerebellar Purkinje cells (Dupere *et al.*, 1996) and antagonise P-, N- and L-type VSCC in a voltage-dependent manner (Norris *et al.*, 1996). Other pharmacological evidence for cation channel block comes from one endogenous role of cytosolic spermine **1** and spermidine **2** as gating molecules for inward rectifying potassium channels (Ficker *et al.*, 1994; Lopatin *et al.*, 1994).



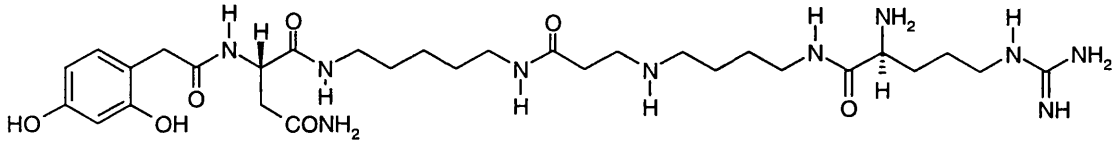
ArgTX-636 **23**



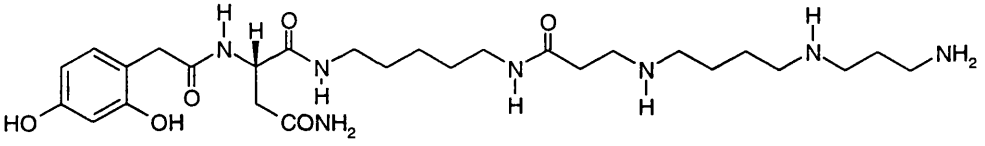
ArgTX-659 **24**



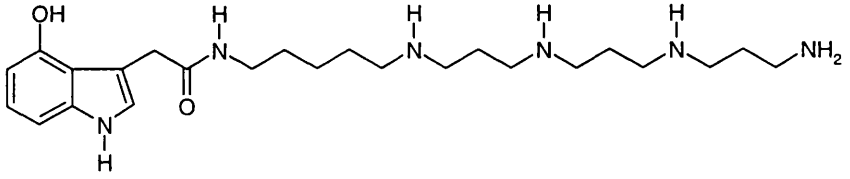
ArgTX-673 **25**



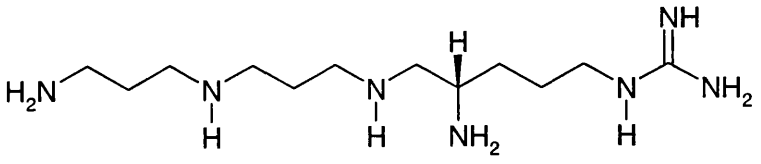
NSTX-3 **26**



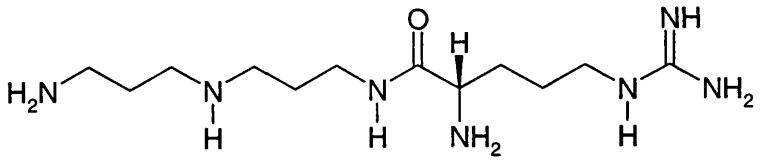
JSTX-3 **27**



CNS 2103 **28**

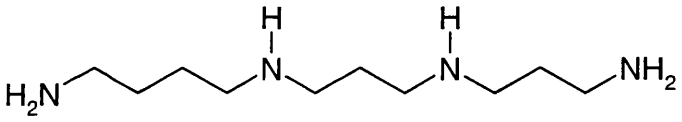


FTX-3.3 **29**

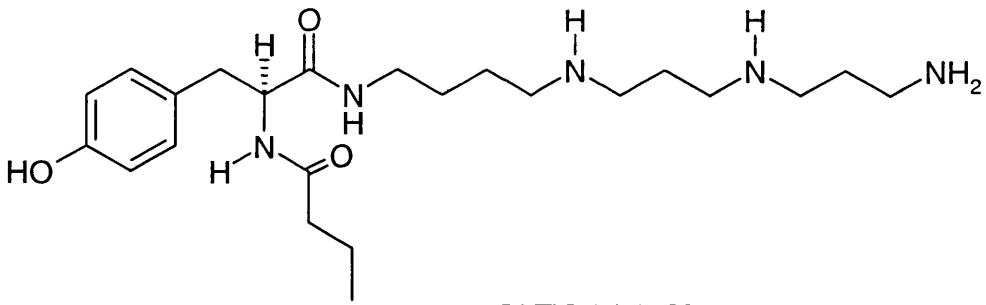


sFTX-3.3 **30**

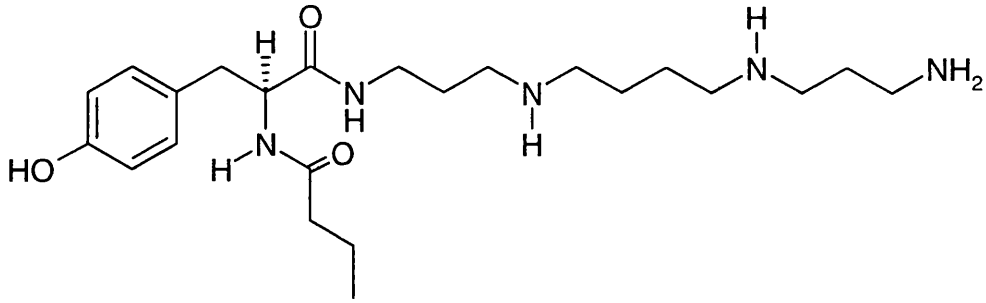
The venom of a solitary parasitic wasp (*Philanthus triangulum*) contains thermospermine **31** (a regioisomer of spermine) conjugated to L-tyrosine in PhTX-4.3.3 **32**. This unsymmetrical polyamine amide is essentially equipotent with synthetic spermine-containing analogue PhTX-3.4.3 **33**. Polyamine amides (philanthotoxins) are potent blockers of the cation channels gated exogenously by nicotine and endogenously by acetylcholine or glutamate. That they are cation channel blockers is not surprising given their structures, essentially fully protonated at physiological pH. The p*K*a data for PhTX-3.4.3 **33** have recently been published: 11.4, 10.4, 9.5 and 8.5, although the third measured p*K*a (9.5) also accounts for the phenolic functional group, and therefore an increase in acidity from pH 10.4 to 9.5 finds both the secondary amine nearer to the tyrosine residue, and the phenoxide of tyrosine gaining protons (Jaroszewski *et al.*, 1996). The p*K*a of phenol is 10.0, and the p*K*a's of tyrosine are 10.1 and 9.1 (and 2.2).



Thermospermine **31**



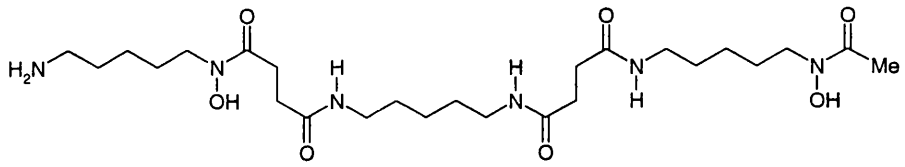
PhTX-4.3.3 **32**



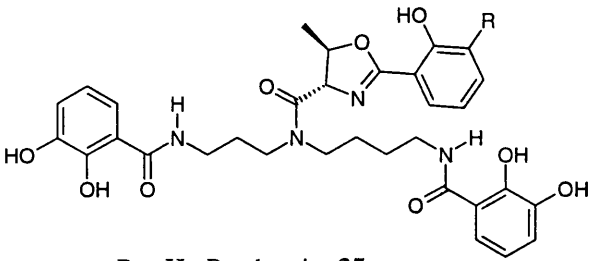
PhTX-3.4.3 **33**

1.8 Polyamine Containing Ion Chelators

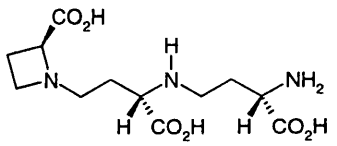
In all forms of life, except for a few species of bacteria, iron plays an important role, principally in metabolic processes where the interconversion between the +2 and +3 oxidation states is utilised in a variety of redox proteins. In the environment, iron generally occurs at the ferric oxidation level which is largely insoluble and hence presents the problem to micro-organisms of how to obtain the iron they require. Microbes have solved this problem by generating iron chelator systems (siderophores) which complex ferric iron allowing it to be accessed (Bergeron, 1984). Many siderophores contain polyamine or polyamide moieties, e.g. desferrioxamine B **34**, parabactin **35** and agrobactin **36**. Phytosiderophores are low molecular weight ion chelating compounds endogenous to plants. They facilitate iron solublisation and transport in a manner analogous to the microbial siderophores. The phytosiderophore nicotianamine **37** (Scholz *et al.*, 1992; Matsuura *et al.*, 1994) is a triamine which contains a primary, a secondary and a tertiary amine functional groups. These polyamine based compounds are potential treatments for haemochromatosis (β-thalassaemia), controlling iron levels (Lee *et al.*, 1993; Giardina and Grady, 1995; Kontoghiorghes, 1995).



Desferrioxamine B **34**



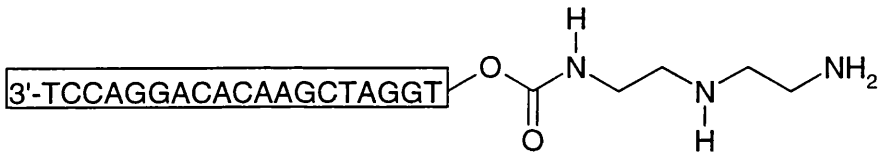
R = H, Parabactin **35**
R = OH, Agrobactin **36**



Nicotianamine **37**

1.9 Polyamine Conjugates as Artificial RNAase and DNAase

Komiyama and co-workers have recently published work on the design and synthesis of synthetic catalysts which hydrolyse RNA with the aim of developing systems which mediate site-selective scission. Originally, the catalysts were based around transition metal chemistry, but recent developments have incorporated oligoamines, such as ethylenediamine, which will efficiently hydrolyse RNAs (Yoshinari *et al.*, 1991). The mechanism of action in this cleavage is by intramolecular acid-base co-operation between an ammonium cation and an uncharged amine. In order to make a site-selective RNA cleaving agent, diethylenetriamine has been linked to a 19-mer piece of synthetic DNA **38**, designed in order that the DNA sequence is complementary with the RNA sequence, adjacent to the desired site for scission.



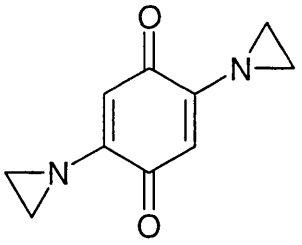
Synthetic RNAase **38**

Ethylenediamine is used here for its acidity, rather than as a metal ion chelator. The p*K*a's of ethylenediamine are 9.2 and 6.5 (Yoshinari *et al.*, 1991), or 9.92 and 6.86 (Albert and Serjeant, 1984). However, "that intramolecular co-operation between two amino residues plays a dominant role is strongly evidenced" with "remarkable acceleration of RNA hydrolysis by simple oligoamines as highly potent catalytic moieties for artificial ribonucleases" (Yoshinari *et al.*, 1991). DNA intercalators (anthraquinones) substituted with metal chelating moieties (ethylenediamines), and complexed to cupric ions, induce the chain cleavage of double stranded DNA (Ihara *et al.* 1994). Other artificial ribonucleases have been designed on a polyamine or amine/imidazole template, with acridine used to effect RNA intercalation (Shinozuka *et al.*, 1994).

1.10 Polyamine-DNA Interactions

In vitro, polyamine interactions with naked DNA cause precipitation, protection from denaturation by heat or shearing and formation of particles (Tabor and Tabor, 1984). Further studies have shown the quantitative relationship behind these interactions demonstrating that DNA is condensed when about 90 % of the negative charges on the sugar-phosphate backbone have been neutralised; an event predicted by the counter-ion theories of Manning (1978). When such experiments are performed in dilute solutions of DNA there is compaction into discrete particles identifiable as rods, spheres or toruses under the electron microscope (Gosule and Schellman, 1976).

When DNA is exposed to dilute solutions of polyamines, transformations are observed between structural forms. Initially, it was assumed that the nature of polyamine-DNA interactions was solely through electrostatic attraction between ammonium cations and the anionic phosphate (Tabor and Tabor, 1964). Recent work has shown that this is not normally the case and that there are preferential polyamine binding sites associated with the major and minor grooves of DNA. The locations of these sites have often proved elusive as the binding is not long lived with frequent migration of polyamines between sites. For this reason, NMR spectroscopic and even X-ray studies have often proved inconclusive. Polyamines do not contain chromophoric moieties and conjugation to chromophores or photoaffinity labels will affect the mode of binding to some extent. Computer molecular modelling has gone a long way in identifying preferential binding sites (Haworth *et al.*, 1991) along with UV spectroscopy and DNA footprinting. In GC rich regions, there is a binding site in the major groove, identified by molecular mechanics and by experiment showing spermine **1** blocks inter-strand cross-linking by 2,5-diaziridinylbenzoquinone **39**, a DNA cross-linker with a preference for a specific GC site (Yuki *et al.*, 1996).



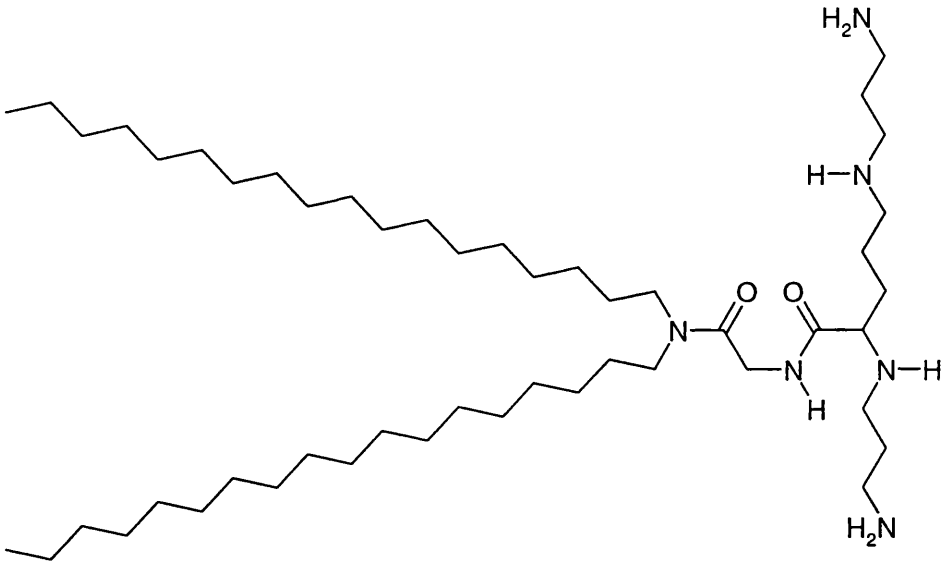
2,5-Diaziridinylbenzoquinone **39**

The formation of hydrogen bonds between DNA bases and polyamines when bound cause bending of the double helix (Feuerstein *et al.*, 1986 and 1990). Higher concentrations of polyamines can cause changes in the helical structure, typically with transformations between Z and B (Behe and Felenfeld, 1981) or B and A (Jain *et al.*, 1989) forms, depending on the exact conditions. These transitions typically occur at polyamine concentrations comparable to those in the intracellular environment. Polyamines, along with histones, fulfil crucial roles defining the structure of chromatin and protecting DNA (Marton and Pegg, 1995). Depletion of nuclear polyamines leaves DNA much more susceptible to digestion by DNAase enzymes suggesting a change in the chromatin structure and relaxation of DNA to a less compacted form (Basu *et al.*, 1992). Histones account for 50 % of the charge neutralisation of chromosomal DNA (Matthews, 1993). Polyamines can provide charge neutralisation for the remainder through either non-specific interactions or interactions at their preferred binding sites. By modulating acylation of the histone and polyamine populations, cells can control the bending of DNA, a process which may be important at times of nucleosome formation and gene expression. Both histone H4 and spermidine are acylated by histone acetyltransferase, in the same region of chromatin. High levels of acylated histone H4 are associated specifically with transcription (Waterborg *et al.*, 1984) leading to the hypothesis that there is a synergistic interaction between acylated histone and spermidine in this process (Matthews, 1993).

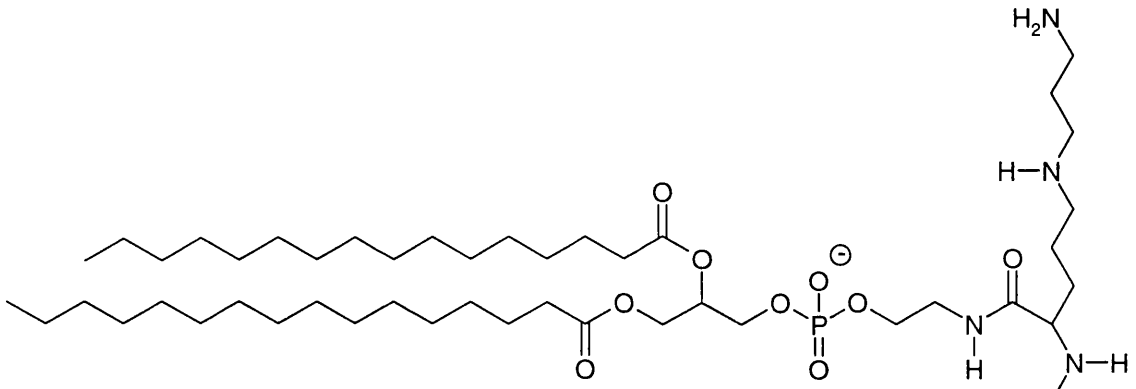
1.11 Gene Therapy

Gene therapy is the correction of a genetic fault (e.g. cystic fibrosis) by delivering DNA containing a correct copy of the defective gene across a cellular membrane and thence to the cell nucleus. Some sort of compaction and charge neutralisation system is required to package the foreign DNA into a suitable form for cellular uptake. Once inside the cell, the DNA must escape the lysosome and migrate to the nucleus where it can be incorporated into the genetic material and express the correct gene.

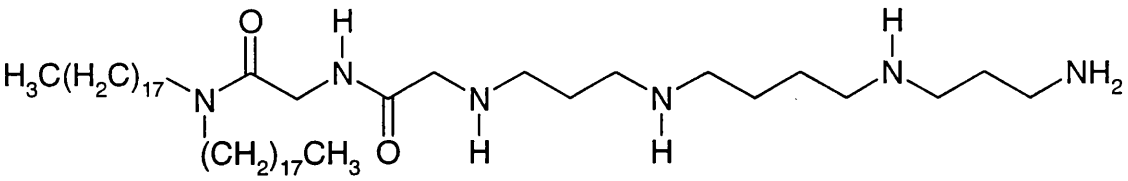
Polyamines have been shown to compact DNA into forms suitable for uptake into cells, but initial studies with spermidine and spermine showed that although the DNA was suitably compacted, the polyamine/DNA interactions were readily reversible making them unsuitable transfection candidates (Behr, 1993). Structure-activity studies with a range of polyamine conjugates have shown that the most suitable systems consist of a polyamine covalently bound to a lipid (lipopolyamines) (Behr *et al.*, 1986; Byk *et al.*, 1997). Dioctadecylamidoglycylspermine (DOGS, Transfectam) **40**, and dipalmitoyl-phosphatidylethanolamine spermine (DPPES) **41** were the first polyamine based lipid (lipopolyamine) gene delivery vectors (Behr *et al.*, 1989); RPR120535 **42** is one of the most recent (Byk *et al.*, 1998).



Transfectam **40**



DPPES **41**

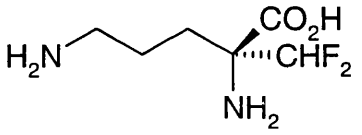


RPR120535 **42**

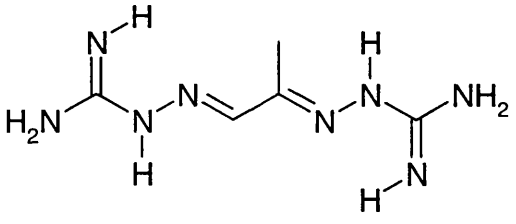
These molecules contain spermine covalently bound to two hydrophobic chains. When mixed with DNA, these polyamine-incorporating vectors cause condensation and formation of self-organised compact nuclear particles with an excess coat of cationic lipid. It is interesting to note that Transfectam **40** does not require the use of chloroquine **12** or an other weak base for successful transfection. In previous non-polyamine transfection systems the presence of such a base has been required to buffer the lysosome and to protect the DNA from degradative lysosomal enzymes. It is possible that the buffering capacity of the modified spermine fulfils this role, an interpretation which is supported by considering the p*K*a values published for Transfectam **40** which are 10.5, 9.5, 8.4 and 5.5. It may indeed be significant that the fourth (lowest) of these p*K*a values corresponds to the internal pH of the acidic lysosome (Remy *et al.*, 1994). For a recently published overview of synthetic polyamine gene delivery agents, see: Miller, 1998.

1.12 Polyamine Conjugates in the Treatment of Cancer

Due to the fundamental roles polyamines play in cellular growth, it is not suprising that polyamine conjugates or compounds which interfere with polyamine pathways are the focus of significant attention as potential anti-cancer agents. Rapidly proliferating cancer cells require large quantities of intracellular polyamines to maintain their growth rate. A first line of attack is the use of inhibitors against the enzymes in the polyamine biosynthetic pathway. Difluoromethylornithine (DFMO) **9**, an inhibitor of the ODC enzyme (Metcalf *et al.*, 1978; Bacchi *et al.*, 1987), showed initial promising activity in animal tumour models, but has failed to show significant activity in clinical trials, either when used alone or in conjunction with other agents. One exception was the combination of DFMO **9** and methylglyoxal-bis(guanohydrazone) (MGBG) **43**, an inhibitor of AdoMetDC, which produced significant responses in patients with glial tumours (Marton and Pegg, 1995). Unfortunately, liver toxicity has proved to be a significant problem with this treatment.



DFMO **9**

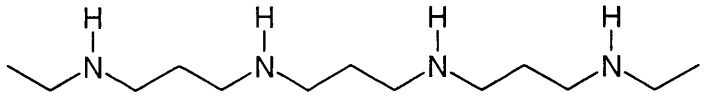


MGBG **43**

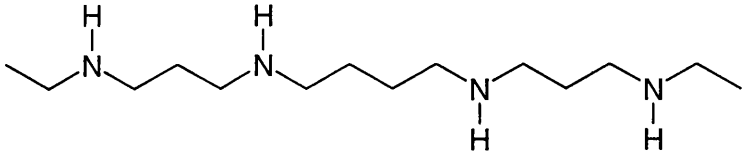
Overall, the problems associated with the use of DFMO and other inhibitors as the key therapeutic agents are associated with the rapid turnover of the target enzymes, making it necessary to administer continuously the inhibitors during treatment, and the resourceful nature of the cell in locating the polyamines it requires. Polyamines can be obtained from the diet and from by-products excreted by microbial flora in the gut.

Spermidine **2** and putrescine **3** are produced from the back conversion pathway from spermine **1** (SSAT, PAO), and also spermine stored in other cells can be exploited. These problems can be minimised by feeding patients on specially produced polyamine-free food, administration of antibiotics to limit the number of microbes in the gut and by inhibiting the metabolic pathway. Possibly a more important role for DFMO **9** is as a component co-administered with drugs which are designed to be taken into cells by the polyamine transporter. The inhibition of polyamine biosynthesis which DMFO **9** causes leads to a corresponding upregulation of the transporter as the cells try to maintain their intracellular polyamine concentrations. This can lead to an enhanced uptake of drug conjugate.

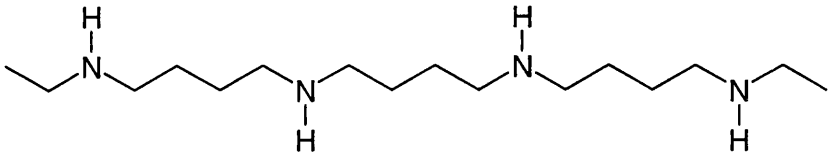
Some of the simplest and most effective synthetic polyamines to show anti-cancer activity have been developed by Porter, Bergeron and their co-workers. Initial activity of *N*-alkylated spermidine (Porter *et al.*, 1982 and 1985) and spermine derivatives (Bergeron *et al.*, 1988) was reported *in vitro* against L1210 leukaemia. These compounds were designed with three aspects in mind: they should be recognised and gain entry to the cell by the polyamine transporter, they should competitively replace endogenous polyamines in the cell, and they should function differently to endogenous polyamines at sites of polyamine activity. Studies were continued with series of triamines and tetraamines with various methylene backbones and patterns of *N*-alkylation (Bergeron *et al.*, 1989 and 1994). The most effective of these compounds were found to be terminally *N*-ethylated tetraamines DENSPM **44**, DESPM **45**, DEHSPM **21** which displayed IC_{50} values of 1.3 μM, 0.2 μM and 0.06 μM respectively over 96 h, in *in vitro* cultures of L1210 cells. DENSPM **44** is the subject of phase I clinical trials against solid tumours (Kramer *et al.*, 1997).



DENSPM **44**



DESPM **45**

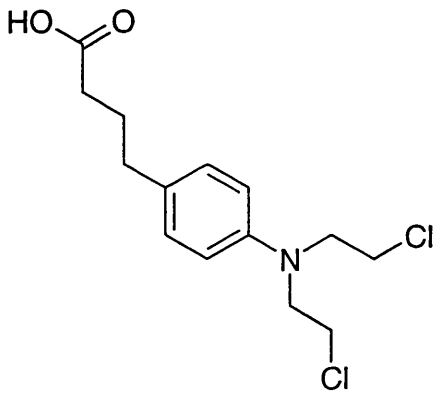


DEHSPM **21**

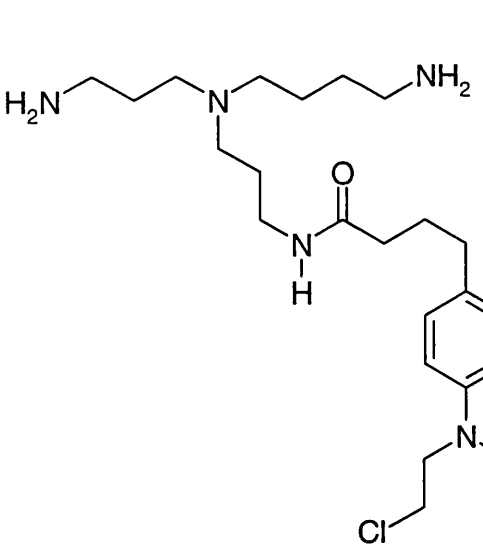
These compounds utilise the transport apparatus and deplete intracellular polyamine pools. Their accumulation can upregulate SSAT increasing the rate of metabolism of spermine **1** or spermidine **2** (Fogel-Petrovic *et al.*, 1993) and reduce the activities of ODC and AdoMetDC. Although there is a significant variation in the time it takes for cellular incorporation, probably due to different affinities to the transporter, over a period of 24 h the total amount of cellular polyamine normalised for the nitrogen content remains constant. If one molecule of (synthetic) tetraamine is incorporated, then one molecule of spermine **1** is metabolised and excreted. It has been demonstrated that these compounds do not have the activity displayed by endogenous polyamines with respect to chromatin and DNA stabilisation. Replacing natural polyamines with DEHSPM *in vitro* left cellular DNA more susceptible to attack by DNAase enzymes in a manner similar to that seen when the cell is totally depleted of its polyamine content. This leads to the conclusion that DEHSPM **21** is not stabilising the chromatin in the way required for normal transcription and gene expression. More recently, the analogous dialkylated triamines have been re-evaluated. Although initially discarded due to lesser cytotoxic activity than corresponding tetraamines, these compounds are again the

subject of experiment in animal models due to their significantly smaller toxicities, a factor important when long durations of treatment are required (Bergeron *et al.*, 1997).

Another approach to the development of anti-tumour compounds is the covalent linking of cytotoxic agents, whose activity is mediated through direct interaction with DNA, to a polyamine. The resulting conjugate will be transported into the cell through the polyamine transport mechanism (if recognised) and the polyamine should further aid DNA binding of the cytotoxic component at its DNA target site. Chlorambucil **46** is a nitrogen mustard-containing compound which is used in the clinic to treat a number of cancers, its mechanism of action is DNA alkylation and hence cross-linking of DNA strands.



Chlorambucil **46**



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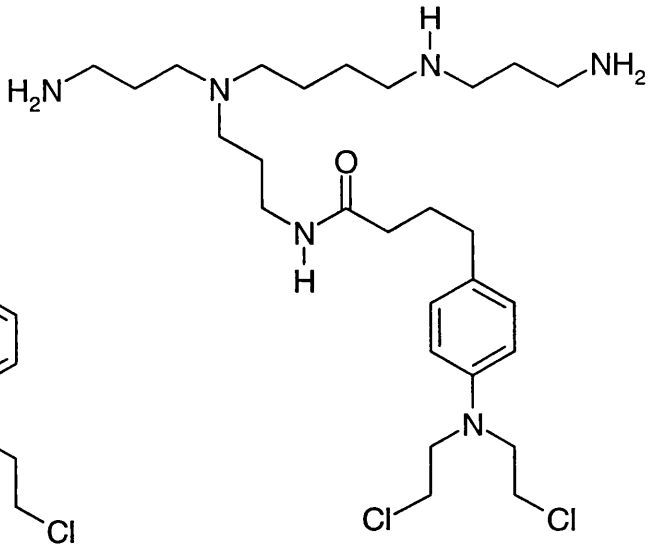
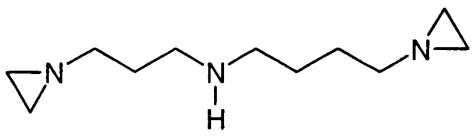


48

Chlorambucil **46** has been conjugated to both spermidine **2** and, spermine **1** resulting in DNA cross-linkers with the potential to carry up to 3 or 4 positive charges at physiological pH (Cohen *et al.*, 1992; Cullis *et al.*, 1995). *In vitro* experiments have established that these polyamine conjugates are recognised by cellular uptake systems; the spermidine conjugate **47** displayed ~35 times more cytotoxicity than chlorambucil **46** alone. These conjugates **47** and **48** alkylate DNA in the same positions as chlorambucil **46** indicating that the polyamine moiety is not affecting the mechanism of alkylation (Cullis *et al.*, 1998). However, *in vivo* studies did not display the high levels of activity predicted by the *in vitro* assays (Cullis *et al.*, 1995).

A more promising approach to the design and synthesis of polyamines containing a reactive functional group capable of DNA alkylation has recently been described by Callery and co-workers (Li *et al.*, 1996). They have prepared spermidine **49** and spermine **50** analogues with the primary amines replaced by aziridine functional groups to give a bis-alkylating agent bound to a polyamine backbone.



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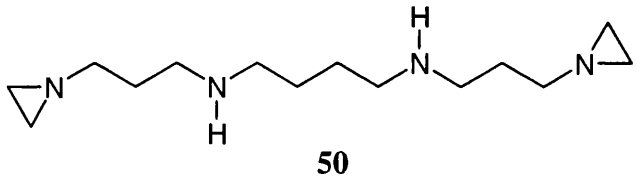


50

Studies showed these compounds to be transported into cells and to cross-link DNA. The *in vivo* activity of spermine analogue **50** was comparable to that shown by other bis-alkylating agents in the same assay, making it a lead compound with the potential for further development.

1.13 Aims

The aims of this research are to produce conjugates of polyamines which have been designed to have potentially medicinally interesting properties. Two key areas have been studied: cytotoxic agents targeted against tumours, and inhibitors of the trypanothione reductase enzyme to treat trypanosomal diseases.

The affinity of polyamines for DNA and their transport mechanisms make them ideal targeting agents for cytotoxic agents with mechanisms of action associated with DNA. The transport mechanisms are known to be both flexible enough to recognise modified polyamines and to be upregulated in rapidly proliferating cancer cells. This gives an enhanced uptake of drug in cancer cells. The polyamine binding to DNA, once in the cell, will bring the conjugated cytotoxic moiety close to its site of action enhancing its activity. In these studies, polyamines have been conjugated to cytotoxic agents which intercalate between DNA base pairs. Conjugates have been prepared by "classical" solution chemistry employing the use of amine protecting groups to ensure efficient, specific synthesis as well as by the more recently developed solid-phase synthetic chemistry techniques.

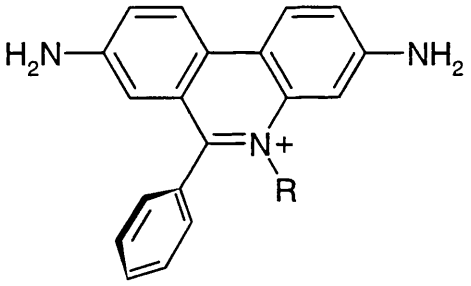
Natural product leads have been used to identify compounds with potential trypanothione reductase activity. Typically, these compounds are polyamines diacylated with cinnamic acid derivatives. In these studies, such analogues have been synthesised in which the 6-membered aromatic rings from the cinnamic acids are linked *via* a diphenyl ether bridge making macrocycles with ring sizes between 23- and 28-members. The synthetic challenges in this work include the reaction of unsymmetrical polyamines in a regiospecific way, the efficient formation of diphenyl ether bonds and the ring closure of various sizes of macrocycle.

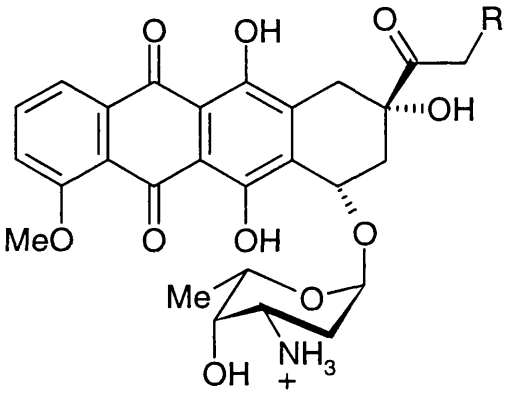
Chapter 2

Polyamine Conjugates of Anthracene- and Acridine-9-carboxylic Acids

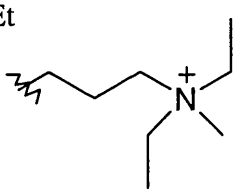
Polyamine Conjugates of Anthracene- and Acridine-9-carboxylic Acids

DNA intercalators are generally planar, aromatic, polycyclic compounds (e.g. ethidium **51** and propidium **52**) which are able to bind between the planar heterocyclic base pairs, perpendicular to the axis of the double helix (Wilson, 1996). This binding is brought about by π-π stacking interactions between the systems of delocalised electrons in the intercalator and in the DNA base rings. In order to create an intercalation site in a DNA duplex, there is a separation of base pairs resulting in the lengthening of the DNA strand and a decrease in the torsional twist, causing unwinding near the location of the intercalation site. These effects are not observed when compounds with groove-binding modes of interaction are exposed to DNA (Wilson, 1996). The changes in shape mean that many intercalating compounds, such as daunomycin **53** and adriamycin **54**, display cytotoxic activities as transcription is impeded and hence cell replication can no longer be performed (Neidle and Sanderson, 1983). The disruption near the site of intercalation is possibly recognised by repair enzymes which cause either single- or double-stranded scission in order to relieve the conformational strain.





51 Ethidium R = Et

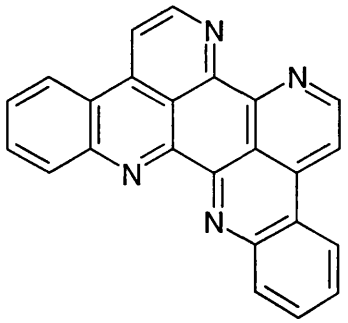
52 Propidium R = 

53 Daunomycin R = H

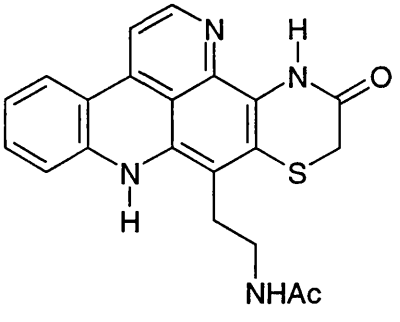
54 Adriamycin R = OH

By attaching substituents to intercalating moieties which point away from the DNA helix, inhibition of DNA associated enzymes can be achieved. Notable examples are the topoisomerase DNA processing enzymes which can interact with intercalator side chains. The function of the topoisomerases (Sinha, 1995; Berger *et al.*, 1996; Withoff *et al.*,1996) is to control the degree of twisting in closed circular DNA either by making a single strand break, in the case of topoisomerase I, or a double strand break, in the case of topoisomerase II and allowing twisting of the strands. Both enzymes operate mainly on supercoiled DNA converting it into different topoisomers, but they will operate on linear DNA in regions exhibiting torsional stress. Whilst there is an intercalator present, topoisomerase II may make a break in the DNA close by, in order to try to relieve some of the excess of strain generated by the intercalation pocket. A three-way complex can then be formed between the intercalator and DNA and by part of the intercalator protruding into one of the grooves interacting with topoisomerase. This ternary complex is stable and prevents the enzyme from rejoining the DNA strand (Lui, 1989; Spicer *et al.*, 1997).

Topoisomerases are known to be overexpressed in many proliferating tumour cells types making them ideal targets for cancer chemotherapy (Heck *et al.*, 1988). The marine natural products eilatin **55** and shermilamine B **56** are examples of pyridoacridine intercalators with inhibitor activity against topoisomerase II (Hagan *et al.*, 1997).



Eilatin **55**

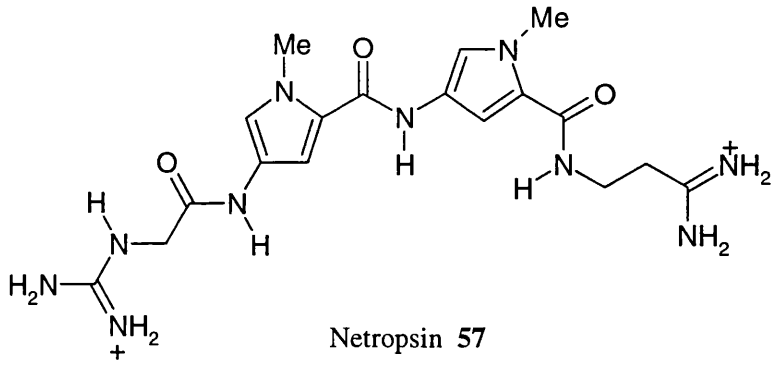


Shermilamine B **56**

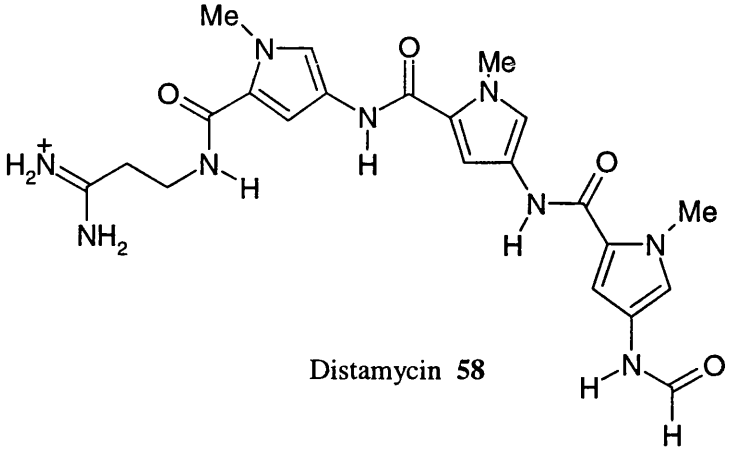
One other way in which compounds can interact with DNA is by binding in either the major or the minor grooves. Compounds which exhibit these modes of binding include polyamines, as discussed in Chapter 1, and structures such as netropsin **57** and distamycin **58** (Wilson, 1996), propamidine **59** (Nunn *et al.*, 1993; Nunn and Neidle, 1995) pentamidine **60** (Sansom *et al.*, 1990; Edwards *et al.*, 1992; Greenidge *et al.*, 1993), DAPI **61** (Barcellona and Gratton, 1996) and berenil **62** (Jenkins *et al.*, 1993).

Groove-binding compounds typically contain amine and ammonium ion groups for hydrogen and electrostatic bond formation. Hydrogen bonds are formed between amines and carbonyl groups on the DNA bases and electrostatic bonding occurs between the ammonium cations and the anions on the DNA sugar-phosphate backbone. There is also the possibility of the formation of van der Waals bonds between moieties such as methyl groups in thymine residues and carbons on the groove-binding chain furthest into the groove. Groove binders tend to be chain-like and, if not flexible like spermine, then they have a crescent shape which will, in general, follow the curvature of the groove.

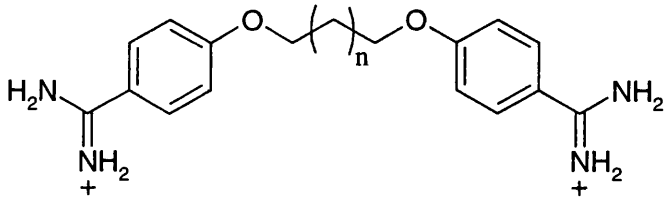
Recently, details have been published of conjugates between groove-binders and anti-cancer agents, such as intercalators and DNA alkylating moieties (Lee *et al.*, 1993; Xie *et al.*, 1995; Wyatt *et al.*, 1997). Although the majority of groove binders are not noted for cytotoxic activity, many do possess specificity for certain DNA sequences and they have also been shown to interfere with both topoisomerases I and II. Site-specific intercalating drugs (Eliadis *et al.*, 1988) have potential for targeting DNA in cancer cells and, by attaching an intercalator, the activity of topoisomerases in these cells may be modulated. An example of such a conjugate is a hybrid of netropsin **57** and the anti-tumour acridine amsacrine **63**. This conjugate **64** has been shown to have groove binding and intercalating modes of action in chromatin preparations, has topoisomerase II inhibitory activity, has reasonable growth inhibition *in vitro,* but displays only moderate *in vivo* activity (Bailly *et al.*, 1992).



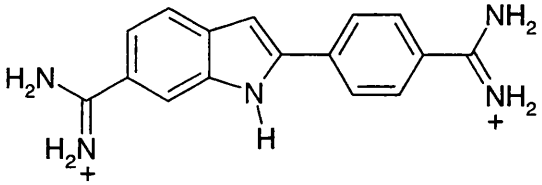
Netropsin **57**



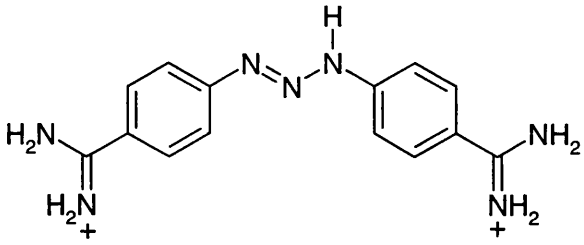
Distamycin **58**



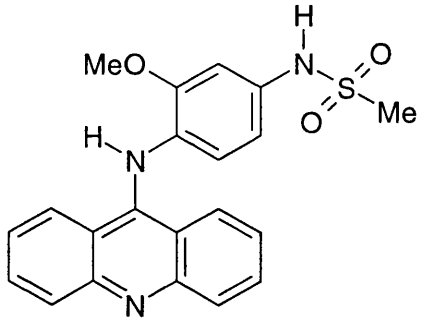
Propamidine n = 1 **59**
Pentamidine n = 3 **60**



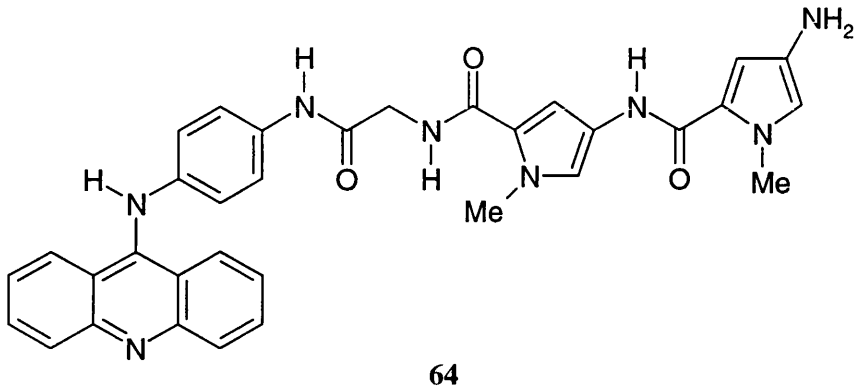
DAPI (4',6-diamidino-2-phenylindole) **61**



Berenil **62**



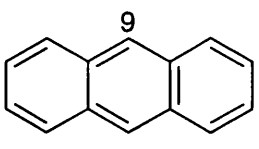
Amsacrine **63**



64

Polyamines are ideal candidates for conjugation with intercalators. As well as targeting the DNA grooves, they are also flexible, possess a dedicated cellular uptake system and there is increasing evidence of their DNA site selectivity (Yuki *et al.*, 1996). Flexibility may be a requirement for efficient bifunctional modes of conjugate-DNA interaction as there is a corner to be turned between the intercalator, perpendicular to the helix, and the groove binder lying in a roughly parallel orientation to that axis. This could be a problem experienced with conjugates containing more rigid groove-binders such as netropsin **57**. The problem of cellular transport could be one reason why netropsin conjugate **64** showed only moderate *in vivo* activity.

The intercalators chosen for this work were the hydrocarbon anthracene **65** and the analogous pyridine derived acridine **66**.



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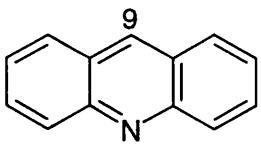
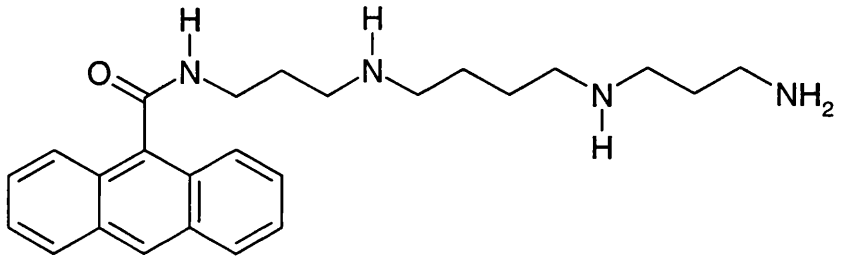


66

The first two targets were conjugates between the 9-position of these aromatics and a terminal amine on spermine, through an amide linkage. These compounds, **67** and **68**, have three positive charges along the polyamine chain at physiological pH making the groove-binding moiety a spermidine rather then a spermine mimic. Initial studies using linear dichroism, circular dichroism and data interpretation facilitated by molecular modelling had been carried out at the University of Bath and with outside collaborators, and these studies showed that the conjugates would indeed show bifunctional DNA binding modes (Adlam *et al.*, 1994; Rodger *et al.*, 1994 and 1995).



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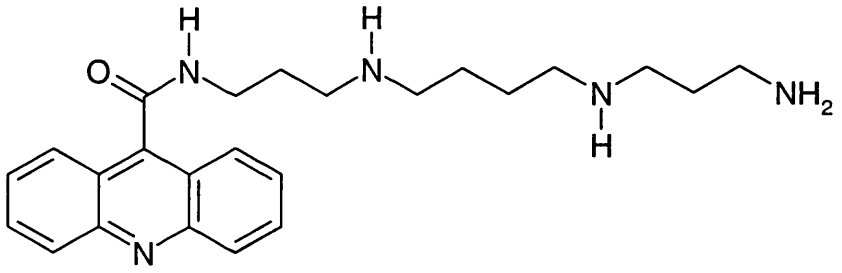


68

In order to synthesise sufficient quantities of the targets **67** and **68** in an efficient manner, careful attention was paid to the polyamine moiety, especially the problem of how to introduce the aromatic chromophore at a specific position. Much synthetic work has been published on building up polyamines with protecting groups on specific amines (Ganem, 1982; Bergeron, 1986; Fiedler and Hesse, 1993; Blagbrough and Geall, 1998; Pak *et al.*, 1998). Usually the synthesis starts with a diamine and the polyamine chain is built from suitably protected fragments, often employing nucleophilic

substitution of halogens, Michael conjugate additions or reductive aminations. Fig 2.1 shows the general method developed by Bergeron and co-workers (Bergeron *et al.*, 1994) for synthesising terminally *N*-ethylated symmetrical and unsymmetrical tetraamines. The mesitylenesulfonyl is used as a protecting group. The proton on the nitrogen in such sulfonamides is sufficiently acidic to be abstracted by NaH generating an anion which will attack electrophilic centres, in this case the carbon adjacent to a halogen in the fragment of polyamine to be added.

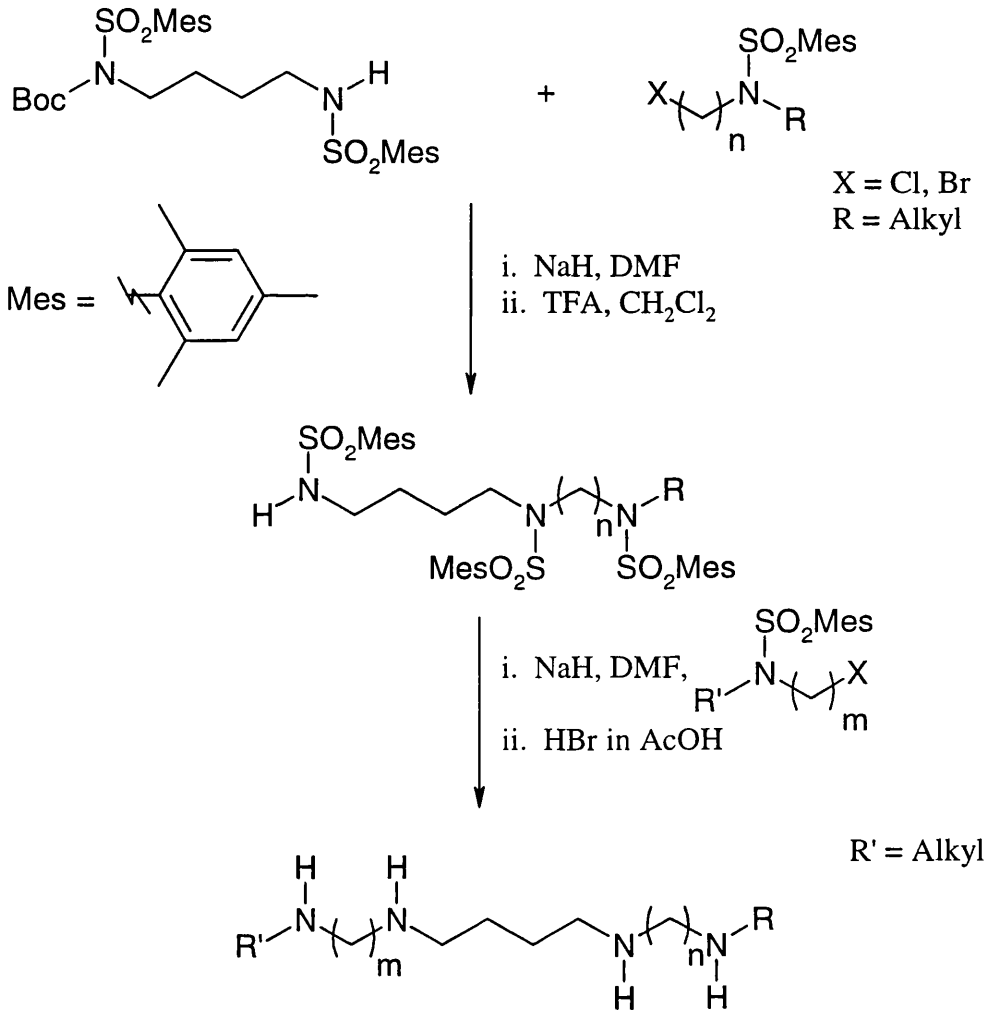


Fig. 2.1

Fig. 2.2 shows the use of Michael additions with acrylonitrile to extend diamines by two propylamine units affording natural and unnatural tetraamines after catalytic hydrogenation of the cyano functional groups (Edwards *et al.*, 1991).

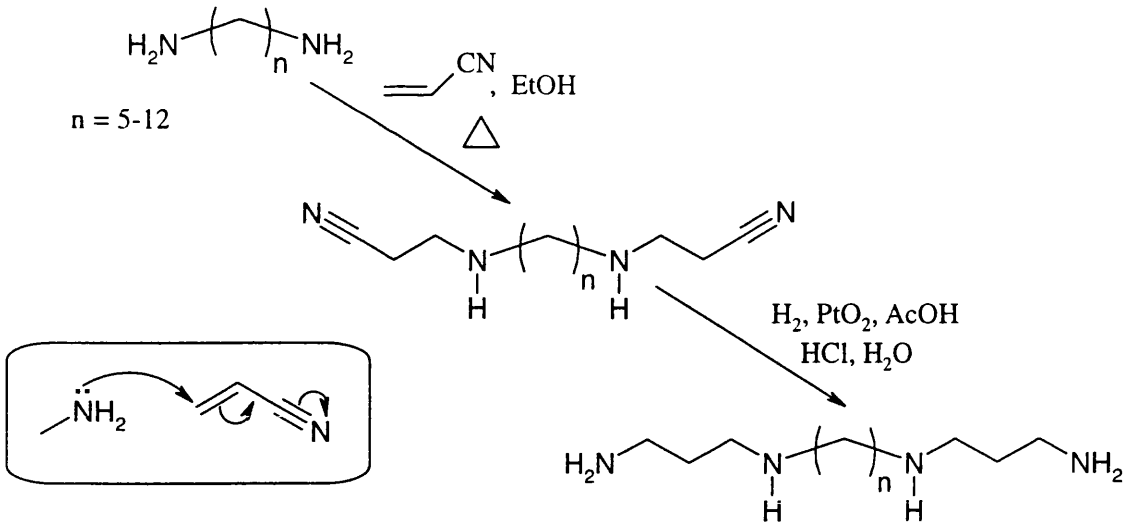

n = 5-12
EtOH
H2, PtO2, AcOH
HCl, H2O

Fig. 2.2

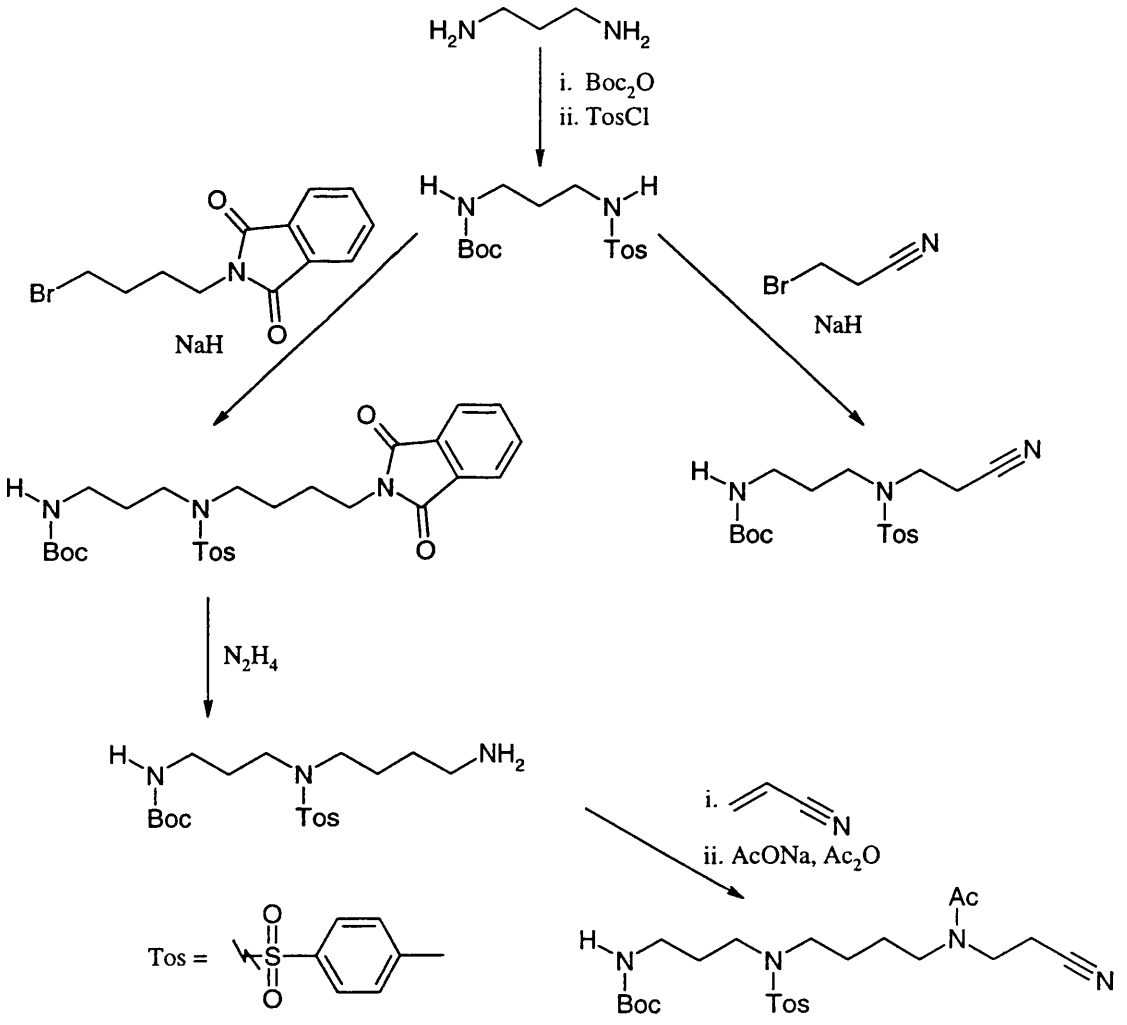

i. Boc2O
ii. TosCl
NaH
NaH
N2H4
i.
ii. AcONa, Ac2O
Tos =

Fig. 2.3

Fig. 2.3 shows the work of Fiedler and Hesse (1993) demonstrating that by careful selection of conjugation methods and protecting groups then polyamines can be built in an orthogonally protected manner allowing specific amine groups to be unambiguously unmasked. Hesse has used these techniques towards the syntheses of many naturally occurring polyamine conjugates and their analogues. These syntheses are time consuming, but sometimes necessary if the spacing pattern of the amino groups is not readily available from natural sources.

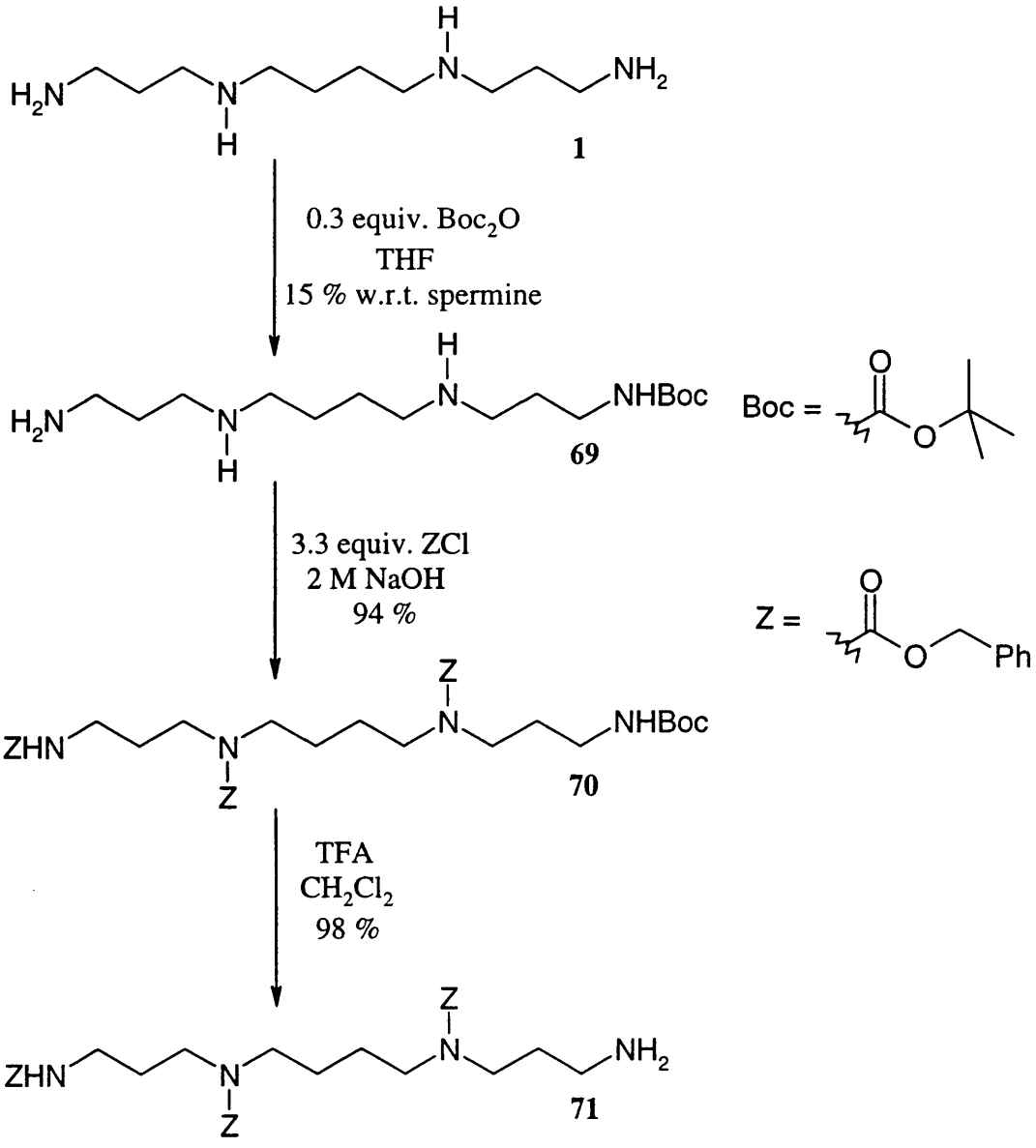


Fig. 2.4

In our initial studies, the polyamine we used was spermine **1** which is readily available (commercially). Rather than starting from a diamine and building the polyamine through the addition of fragments with protecting groups already in place, a strategy for their selective introduction to spermine was developed. Fig. 2.4 shows the strategy for synthesising N^1, N^2, N^3-tri-Z-protected spermine **71** leaving one terminal primary amine ready for acylation. In the first reaction, a Boc group is introduced on one primary amine. This reaction relies on the greater nucleophilicity of primary amines over nucleophilic, but sterically hindered secondary amines.

A threefold excess of spermine **1** was reacted with of Boc anhydride in THF, the excess of polyamine being added in order to favour monoacylated products over di- or triacylated products. Spermine **1** was initially dissolved in THF and the solution cooled to 0 °C before the addition of Boc anhydride in order to ensure that there was no rapid rise in temperature due to any exothermic reaction. A significant temperature rise could affect the selectivity of the acylation leading to a greater proportion of acylated (carbamoylated) secondary amines. After an hour at this temperature the reaction mixture was stirred for a further 18 h at 20 °C, to ensure complete reaction. After work-up, TLC, visualised by dipping the plate in acidic ninhydrin reagent, showed unreacted spermine on the baseline, one major product spot and three minor products. In order to separate the desired product from N^2-Boc-spermine and di-Boc products, careful chromatography was required. Due to the highly basic nature of these compounds and the acidity of the hydroxyl groups on silica gel, if column chromatography is attempted using mixtures of organic solvents then the material either streaks or sticks to the baseline. Addition of a base to the eluant solves this problem as the added base competes with the product for the acidic sites effectively deactivating them. Conc. aq. NH_3 was found to give the best results in TLC experiments, and, when used in the

eluant for flash column chromatography, good separation was achieved. In order to separate the N^1-Boc-spermine 10:4:1, then 6:4:1 and finally 4:2:1 (CH_2Cl_2/MeOH/conc. aq. NH_3) were used to give the compound **69** in 15 % yield with respect to spermine.

N^1-Boc-spermine **69** was then reacted with 3.3 equiv. benzyl chloroformate for 18 h under Schotten-Baumann conditions. The reaction mixture was extracted with CH_2Cl_2 and the residue from the extracts was shown to be the tetra-protected polyamine **70** and it did not require any further purification by TLC and NMR. The Boc group was removed by the action of TFA in a 1:1 mixture with CH_2Cl_2 at 0 °C. The reaction went cleanly and after evaporation of the solvents *in vacuo* the residue was purified by flash column chromatography, again using conc. aq. NH_3 in the eluant, to yield N^1, N^2, N^3-tri-Z-spermine **71** in quantitative yield (Fig. 2.4).

In order to add the intercalating aromatic chromophore to the protected polyamine, 9-anthracene and 9-acridinecarboxylic acids (**72** and **73**) were used. There are a plethora of ways to make amide bonds in the literature mostly stemming from peptide syntheses (Bodansky *et al.*, 1976; Bodansky, 1984 provides a good overview). For this synthesis, the commonly used dicyclohexylcarbodiimide (DCC) was used in a solution containing a catalytic amount of 1-hydroxybenzotriazole (HOBt). This method has the advantage that the major by-product from the acylation is dicyclohexylurea (DCU) which has low solubility so the majority of it can be easily removed from the reaction mixture at the end of the reaction by filtration. This simplifies the subsequent chromatographic purification of the amide product. After recovering the desired amides, the final step required is to remove the Z protecting groups. This was attempted by the commonly used procedure of hydrogenolysis in the presence of a catalytic amount of Pd adsorbed onto carbon. This method of Z removal should be quantitative and yield clean products after filtration of the reaction mixture to remove the catalyst.

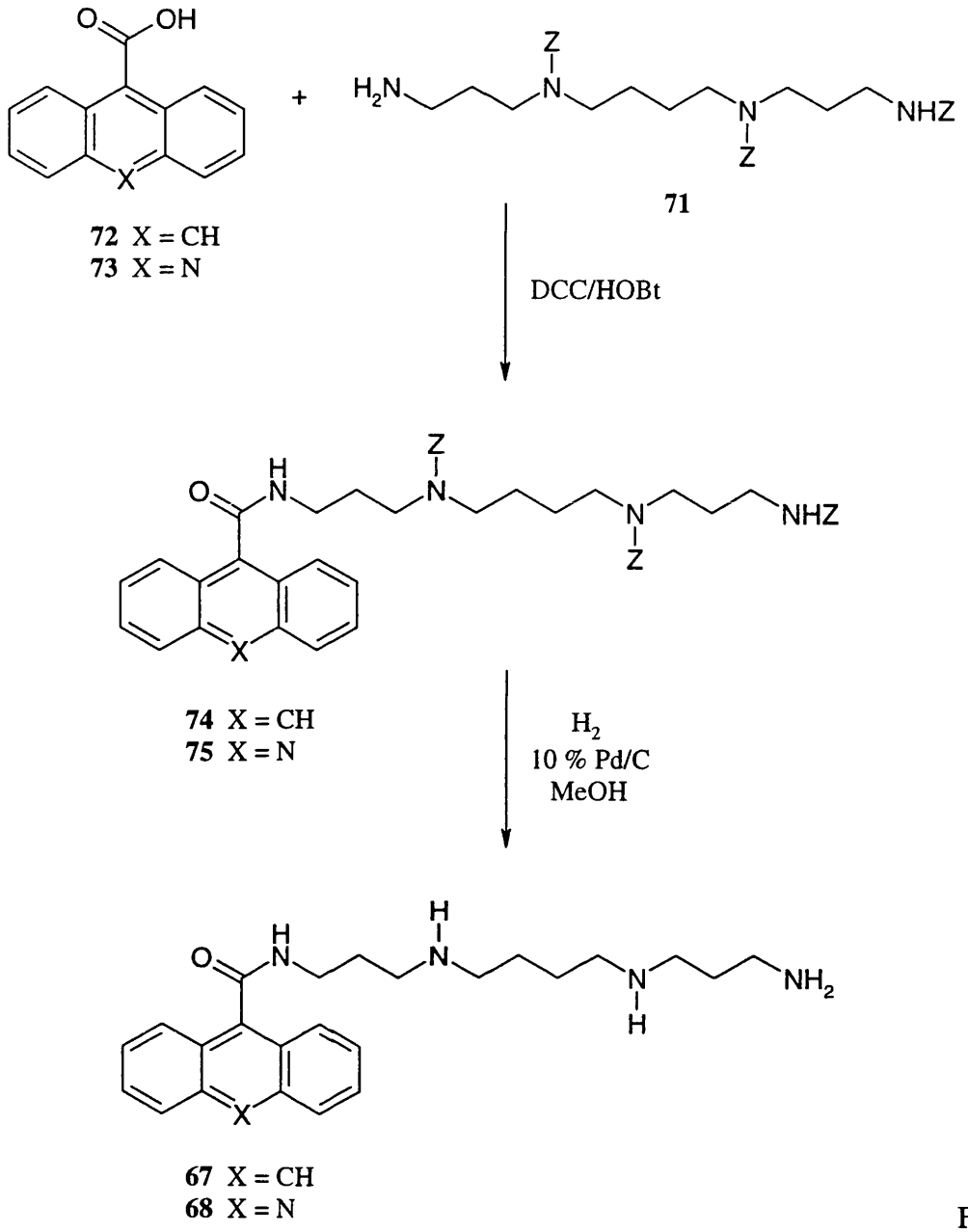


Fig. 2.5

Tri-Z-spermine **71** was coupled to anthracene-9-carboxylic acid **72** using DCC as the coupling (dehydrating) agent in the presence of a catalytic amount of HOBt (Fig. 2.5). The reaction was carried out with equimolar quantities of the acid and amine in CH_2Cl_2 and was seen to be progressing within 30 min as a white precipitate of DCU was formed. After 18 h, TLC showed one major product which was visible with both UV light and ninhydrin reagent and was also less polar than either of the starting materials, of which there were only small amounts remaining. That the product **74**

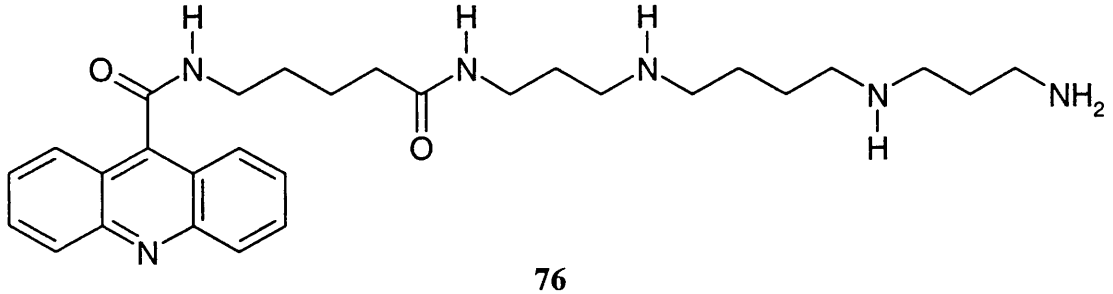
stains with ninhydrin despite the fact that the amines within it have all been functionalised to amides is probably due to decomposition (likely deprotection) or possibly some amide hydrolysis of the compound when the TLC plate is heated.

The desired product **74** was isolated in a good yield (76 %) by flash silica column chromatography, in this case without the need for NH_3 in the eluant as all the amine groups were now masked. The Z protecting groups were removed by hydrogenolysis at atmospheric pressure in the presence of catalytic 10 % Pd on C. After 12 h of reaction, TLC analysis showed there to be starting material still remaining and it was therefore necessary for the reaction mixture to be exposed to H_2 for a further 12 h before the reaction was complete. It had been initially hoped that this reaction would yield pure product after the careful purification of the starting material in order to avoid a potentially difficult purification of this triamine **67**. The TLC after work up showed that a purification step was required so the residue was successfully subjected to column chromatography and the residue from the column was lyophilised. The desired product **67**, isolated in 93 % yield, was a white foam found to be highly hygroscopic. The structure of the product was confirmed by ^{1}H and ^{13}C-NMR and by both low and high resolution FAB-MS. However, it was not possible to get an elemental analysis of C, H and N with sufficient accuracy. This is found to be the case with polyamine conjugates generally. They are hygroscopic and will react with carbon dioxide in the air to give carbonic acid salts. Both of these factors account for the disturbance of elemental composition.

Coupling acridine-9-carboxylic acid **73** with amines is more problematic than the analogous anthracene reactions due to the presence of both a tertiary amine (a pyridine nitrogen) and a carboxylic acid group. These functionalities act in a zwitterionic fashion giving the compound a partially ionic character which makes it particularly hard to solubilise in convenient organic solvents. After investigating a range of solvents DMF

appeared to be the best, although solubility was still limited. In DCC type coupling reactions, the concentration of the reaction mixture needs to be high to ensure fast, efficient reaction. Rather than using a large quantity of DMF to solubilise all of the acridine-9-carboxylic acid **73** and ending up with low concentrations of this and the other reagents, a suspension was made so that a small quantity of the acid would go into solution and react to give a soluble product and then more acid would be solubilised to replace that which had reacted. The reaction was carried out over 48 h with TLC monitoring after which time most of the starting materials appeared to have been consumed. After work up and chromatography, the major product was shown to be the desired product **75**, isolated in 53 % yield. This conjugate was deprotected by catalytic hydrogenation over 24 h and the required target was isolated in 91 % yield after column chromatography. The product **68** was a buff coloured foam after lyophilisation and, as with the anthracene analogue **67**, was found to be hygroscopic.

A potential problem with these analogues relates to how much of the polyamine is in contact with the DNA groove. There is a turn to be made between the intercalator perpendicular to the helical axis and the groove running in a direction parallel (and around) this axis. The polyamine will be flexible enough to turn into the groove, but there may not be an optimal interaction particular between the charged secondary amine closest to the chromophore. In order to alleviate this potential problem, another target **76** was designed incorporating a spacer between acridine and spermine.



76

Initially, a 5 carbon spacer was chosen, this residue being derived from the amino acid 5-aminopentanoic acid (5-aminovaleric acid). This unit should be long enough to show a measurable increase in activity through tighter DNA binding if a linear spacer is required without being too long and causing unfavourable entropic effects.

To synthesise this target **76** a scheme was proposed (Fig. 2.6) where the amine of the 5-aminopentanoic acid **77** is Boc protected **78** and then the free acid is coupled to tri-Z-spermine **71**. TFA is then used to remove the Boc group from conjugate **79** and the chain extended polyamine **80** is then ready to react with acridine-9-carboxylic acid **73** in the same manner described in Fig. 2.5.

Due to the acidic and basic natures of the functionalities, 5-aminopentanoic acid **77** is also zwitterionic (like **73**) giving rise to solubility problems. THF was chosen as the solvent to carry out the Boc protection step as the starting material has some solubility in it, and the product is freely soluble. Boc anhydride was added to a suspension of amino acid in THF and the reaction was stirred, with TLC monitoring, for 48 h after which time all of the starting material appeared to have been consumed. The predominant product was separated by column chromatography, in 82 % isolated yield, and the proton and carbon NMR spectra clearly showed the incorporation of the Boc group. At δ 1.40 ppm a singlet in the ^{1}H-NMR integrated for 9 protons corresponding to the methyl protons in the butyl group and in the ^{13}C-NMR signals at δ 28.3, 79.0 and 156.3 ppm corresponded to the methyl, tertiary and carbonyl carbons. This product **78** was coupled over 48 h in 38 % yield to tri-Z-spermine **71** using DCC and catalytic HOBt to give the fully protected chain extended polyamine **79**. This moiety was then subjected to TFA in CH_2Cl_2 at 0 °C in order to remove the Boc group and unmask the primary amine **80**, reaction being evident from the increased polarity of the product on the TLC plate and its enhanced colour when stained with ninhydrin. NMR experiments confirmed the deprotection reaction had cleaved the Boc group in an isolated yield of 88 % after column chromatography.

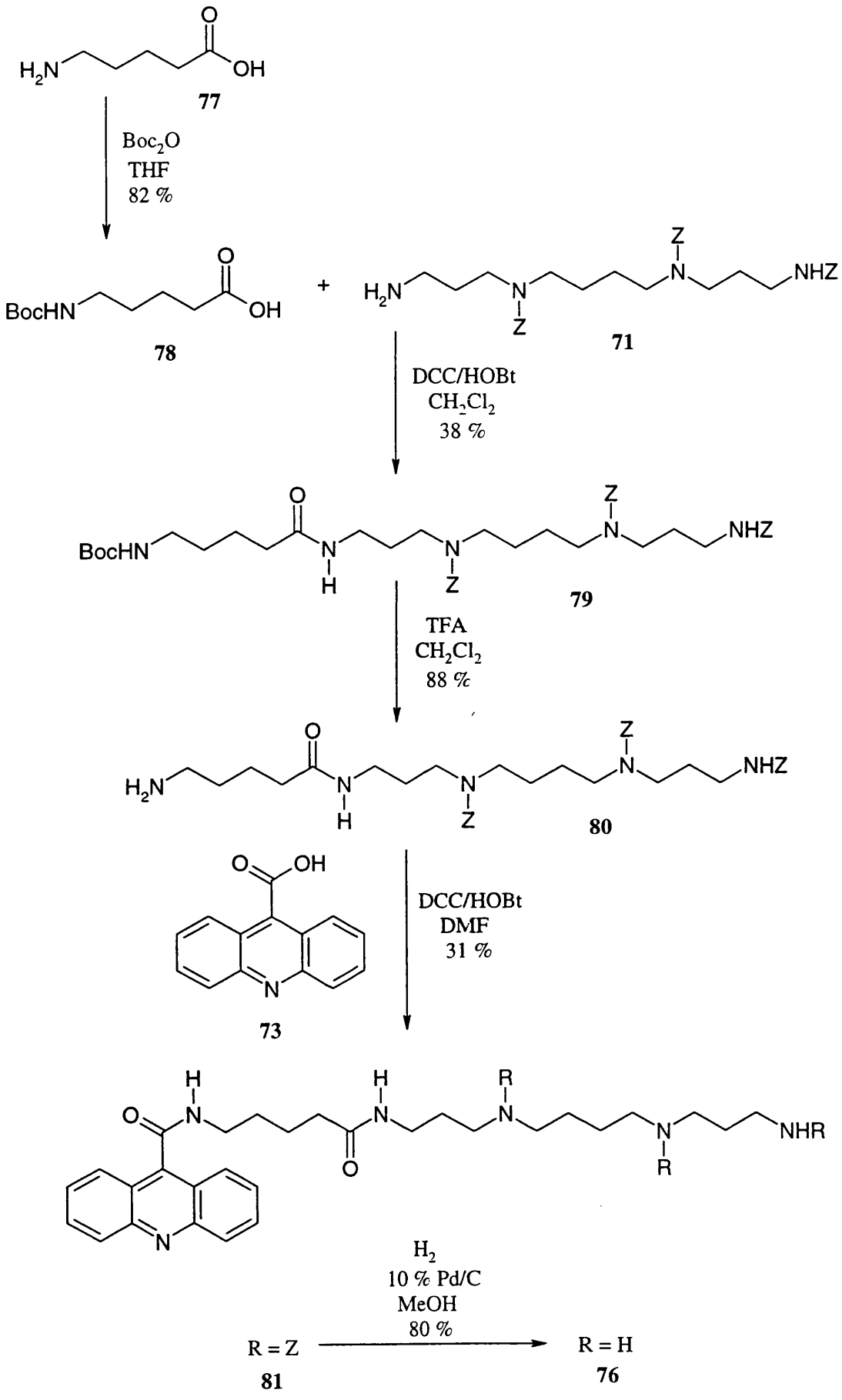
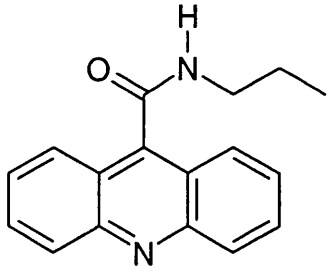

77
Boc2O
THF
82 %
78
71
DCC/HOBt
CH2Cl2
38 %
79
TFA
CH2Cl2
88 %
80
73
DCC/HOBt
DMF
31 %
H2
10 % Pd/C
MeOH
80 %
R = Z
81
R = H
76

Fig. 2.6

The chain extended polyamine **80** was then coupled to acridine-9-carboxlic acid hydrate **73** in DMF with DCC and catalytic HOBt (0.05 equiv.). The reaction was run for 48 h with TLC monitoring and after work-up was purified by silica gel chromatography to give amide **81** in 31 % yield. This Z-protected intermediate was then subjected to hydrogenolysis to unmask the amine groups and the desired target **76** was isolated as a buff coloured hygroscopic foam in 80 % yield after chromatography.

The synthetic conjugates were initially assessed for cytotoxic activity by M. A. Qarawi at the University of Bath. The assay was *in vitro* against B16 murine melanoma cells using the MTT procedure first reported by Mosmann (1983) (Alley *et al.*, 1988). The assay was carried out using 8 x 12 well microtitre plates which could be read by a spectrophotometer. Each row of 8 wells contained the same configuration of cells and/or drug allowing for eight independent determinations per experiment. Rows were set up containing either cells in media or polyamine conjugate in media to act as controls during spectrophotometric measurement and the remaining rows contained cells and polyamine amide at various concentrations. The cells were incubated with the polyamine compounds, typically for 48 h, and were then washed. The cells were then incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 3 h. Cells which remain viable at this time metabolise the MTT to a formazan derivative which has a blue colour. After incubation, DMSO was added to solubilise all of the formazan and photometric analysis was used to assess the amount of formazan present and hence the number of viable cells. To demonstrate that activity seen in the assay was due to bifunctional modes of interaction of the covalently linked conjugates also assayed were spermine, anthracene and acridine-9-carboxylic acids (**72** and **73**) and 1:1 mixtures of the two acids with spermine **1**. The results of the assays are shown in Table 2.1.

Also synthesised was a model amide **82** which is a conjugate of propylamine and acridine caboxylic acid, prepared by DCC/HOBt coupling between the acid and the amine. It was designed to help assess the cytotoxic activity due to the acridine moiety and hence its strength of interaction with DNA. In measuring this, the significance of attaching the polyamine moiety can then be gauged.



82

Table 2.1 Results of the MTT assay

Compound	Mean EC_{50} (M)	SE (±)
9-anthracenecarboxylic acid **72**	$>1.2x10^{-3}$	-
9-acridinecarboxylic acid hydrate **73**	$>8.0x10^{-4}$	-
spermine **1**	$4.5x10^{-4}$	$1.8x10^{-4}$
1:1 spermine:9-anthracenecarboxylic acid	$2.6x10^{-4}$	$8.0x1^{-5}$
1:1 spermine:9-acridinecarboxylic acid	$3.9x10^{-4}$	$8.0x10^{-5}$
9-anth-CO-3.4.3 **67**	$2.0x10^{-5}$	$7.0x10^{-6}$
9-acrid-CO-3.4.3 **68**	$5.4x10^{-6}$	$1.1x10^{-6}$
9-acrid-CO-4-CO-3.4.3 **76**	$1.9x10^{-6}$	$2.7x10^{-7}$

Table 2.1 shows the concentration of each compound (EC_{50}) required to kill 50 % of the cells exposed to it.

The synthesised conjugates showed greater activity than either of their component parts either alone or as a mixture. Of the conjugates, the acridine compounds showed greater activity than the anthracene conjugate and of these, the compound with the five carbon spacer showed the best activity. This preliminary study shows the compounds to be cytotoxic, but gives no information about the precise mode of cytotoxic action of these compounds. Further studies are required to determine activity against a wider range of cell lines, *in vivo* activity and overall toxicity, interaction with the polyamine transporter and activity against topoisomerase enzymes.

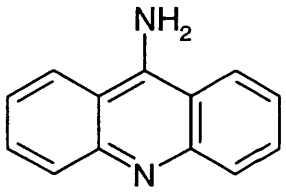
Nevertheless, this initial evidence of the increase in cytotoxicity with more binding modes (both intercalation and groove-binding) is proof that this approach has application in the development of new cytotoxic compounds and is of importance towards the development of new potential chemotherapeutic agents. In these initial studies, we have devised a route for the controlled preparation and purification of unsymmetrical polyamines and their amides which allows the terminal conjugation of a variety of different species to a primary amine. These results in the preparation of novel conjugates of polyamines and DNA intercalators will be used in the design and synthesis of more cytotoxic polyamine conjugates. For example, it is known that 9-aminoacridines are more cytotoxic than acridines bearing other simple substituents. It would be of interest to make further conjugates without the amide, with the polyamine directly attached to the acridine through an amine bond.

Chapter 3

Polyamine Conjugates of 9-Aminoacridine

Polyamine Conjugates of 9-Aminoacridine

Aminoacridine derivatives have been used in clinical medicine since before the turn of the 20th century (Denny *et al.*, 1983). They first came to light as by-products of the aniline dye industry when they were found to have anti-malarial and anti-bacterial activity (Denny *et al.*, 1983). In the 1930's and 40's aminoacridines were found to bind preferentially to DNA and RNA, and certain derivatives were introduced as biological stains. Around this time, 9-aminoacridines were found to have some activity as growth inhibitors of certain tumours (Denny *et al.*, 1983). From this time onwards, there have been many research programmes to develop 9-aminoacridine derivatives as cytotoxic and anti-proliferative therapies.



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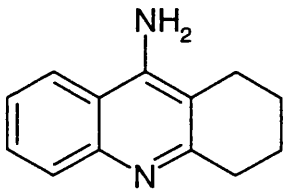
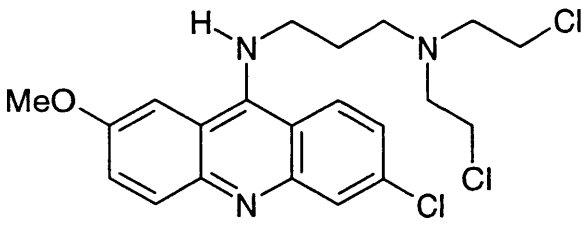


Tacrine **84**



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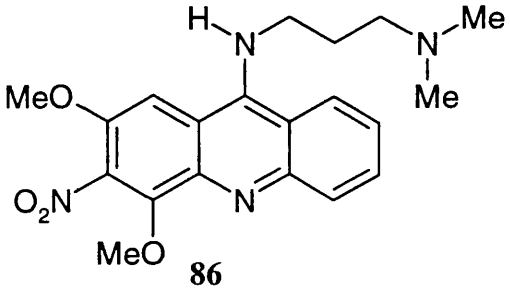

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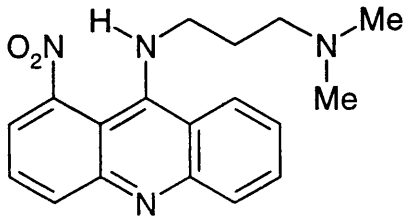


Nitracrine **87**

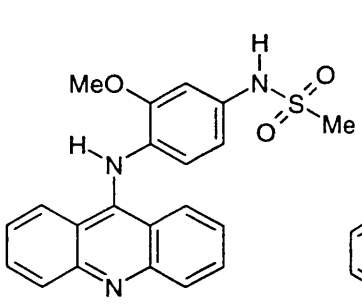
Conjugation to nitrogen mustards gave a range of DNA alkylating agents (e.g. **85**) in which the high affinity of the acridine for DNA brings the mustard group (a β-

haloamine; an aziridinium ion precursor) close to its site of action (Denny *et al.*, 1983). However, the clinical utility of such compounds has been precluded by their high mutagenic activity. Another series of 9-aminoacridines which underwent much evaluation, particularly in Poland, were those with a 1-nitro substituent (e.g. nitracrine **87**) (Denny *et al.*, 1983). These compounds were shown to have both reversible and a smaller proportion of irreversible interactions with DNA, indicating some form of covalent bonding occurring. The precise nature of this interaction has not been elucidated. Clinical trials involving nitracrine **87** have been undertaken in both Poland and the USA, but ended up rejecting its usage due to issues of toxicity. Analogues of these compounds (e.g. **86**) continue to be investigated by Barker and co-workers (Monge *et al.* 1994a and b).

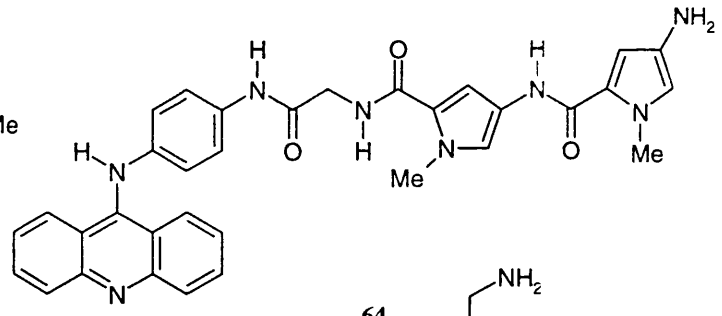
9-Aminoacridine has also been conjugated to DNA groove-binders to give analogues (**64** and **88**) with site specific modes of binding (Eliadis *et al.*, 1988;Bailly *et al.*, 1992), however these compounds have yet to display good *in vivo* activity.

Another 9-aminoacridine analogue with therapeutic interest is the tetrahydro derivative tacrine (**84**). This compound has been shown to be beneficial in the treatment of Alzheimer's disease and other forms of neurodegeneration. The mechanism of action is the inhibition of cholinesterase, hence maintaining the level of the neurotransmitter acetylcholine (Dawson, 1989; Eagger *et al.*, 1991).

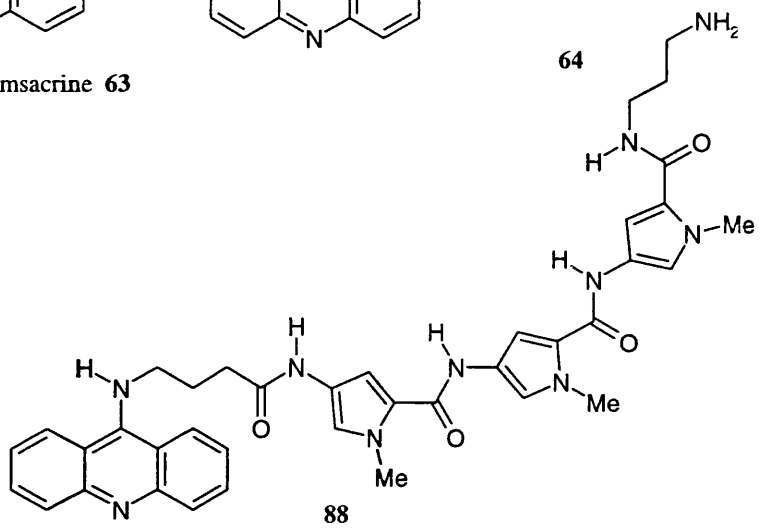
To date, medically the most successful 9-aminoacridine against cancer is amsacrine **63**. This compound has emerged from an extensive programme of synthesis and evaluation under the direction of Cain in New Zealand. The results from this work have largely been published in *J. Med. Chem.* from the late 1960's onwards (for an overview, see: Denny, 1993) and studies with this 9-aminoacridine conjugate are continued today by Denny and co-workers. Amsacrine has successfully completed trials and is used in the clinic in New Zealand against leukaemia (Arlin, 1983).



Amsacrine **63**

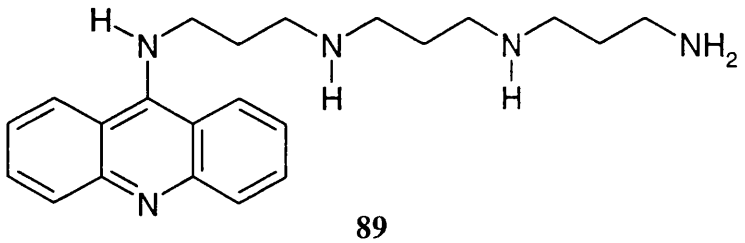


64



88

The studies on amsacrine **63** and its analogues established that they intercalate into DNA and that the strength of this interaction directly correlates with anti-tumour activity both *in vitro* (Ferguson and Baguley, 1981) and *in vivo* (Baguley *et al.*, 1981). The methanesulfonamide group is important for water solubility and stability of the compound as well as for biological activity (Atwell *et al.*, 1972). The 3'-methoxy group is important for anti-tumour activity. If it is moved to the adjacent 2'-position then this analogue is completely inactive against L1210 leukaemia cells *in vitro* (Cain *et al.*, 1975). It has been predicted that, when bound to DNA, the anilino ring protrudes into the minor groove and the methoxy substituent points tangentially away from the helical axis (Wilson *et al.*, 1981). In this position it is free to interact with adjacent proteins such as the topoisomerase enzymes. Amsacrine **63** has been shown to be an inhibitor of topoisomerase II trapping the cleavable complex. Studies have shown that, in close analogues of amsacrine, the ability to stabilise this complex has more importance than simple DNA binding affinity for cytotoxicity.



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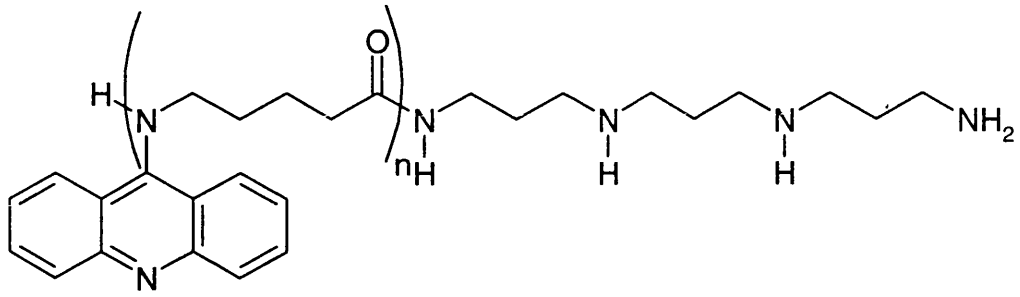
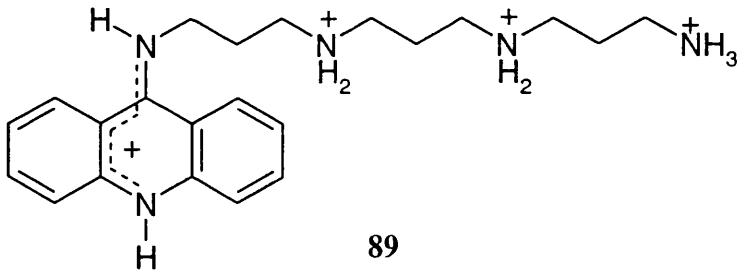


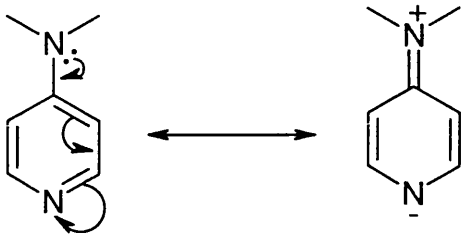
n = 0 **89**
n = 1 **90**
n = 2 **91**

The compounds described in Chapter 2 were 9-carbonyl anthracene or acridines **67**, **68** and **76**, families of compounds not specifically noted for their anti-tumour activity. In order to produce compounds with enhanced cytotoxic activity compounds **89-91** were designed without the amide linkage, but instead having a secondary amine linking acridine with a polyamine. These compounds are 9-aminoacridines. At physiological pH, they will carry close to four positive charges, three on the amino groups mimicking spermidine **2** and one associated with the acridine ring nitrogen and also the secondary amine at the 9-position. It is not clear exactly where the majority of this positive charge resides as there is easy flow of electrons through the aromatic system, and especially around the central pyridine ring of the acridine (c.f. the conjugated system of the nucleophile 4-*N*,*N*-dimethylaminopyridine, DMAP **92**).

As with the 9-carbonyl polyamine conjugates (**67**, **68** and **76**), consideration was given to flexibility in the region of linkage. Again, targets were therefore proposed with a five carbon spacer **90**, and also a ten carbon unit **91** was included to see if increasing the length of the spacer provided any advantage in binding affinity to DNA and therefore in cytotoxicity.

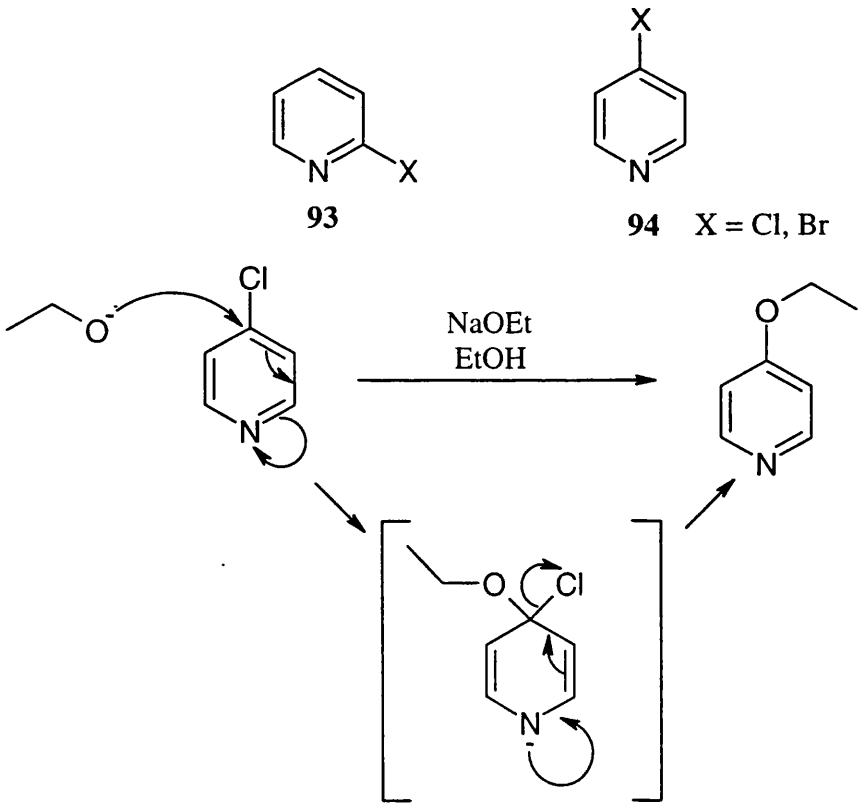


89



92

The syntheses of these compounds were designed bearing in mind that, in acridine, the nitrogen at the 10-position (the pyridine nitrogen) will activate suitable leaving groups at the 9-position for nucleophilic substitution. Such reactions are well known for both 2- **93** and 4- **94** (but not 3-) halo substituted pyridines, the ring nitrogen temporarily stabilising the anionic intermediate (McMurry, 1992) (Fig. 3.1).



93 **94** X = Cl, Br

stabilised anion

Fig. 3.1

9-Chloroacridine **96** would be an ideal starting point for the synthesis, but it has low stability once synthesised and therefore the more stable, but still sufficiently reactive 9-phenoxyacridine **97** was used.

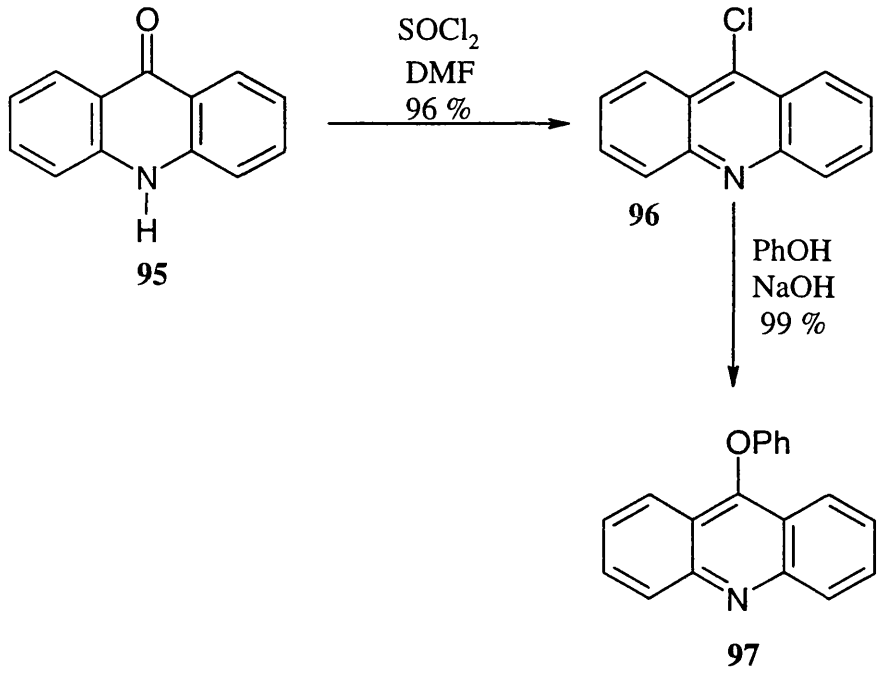
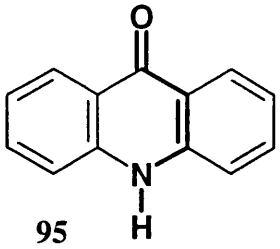


Fig. 3.2

Substitution reactions with 9-phenoxyacridine **97** have been previously described in the literature (Dupre and Robinson, 1945; Eliadis *et al.*, 1988) for a number of nucleophiles, including amines. However, there is no precedent for such reactions employing Z-protected di- or polyamines as the nucleophiles. For this substitution, 9-phenoxyacridine **97** is simply gently heated in phenol, as the solvent, together with the required nucleophile. For the first target **89**, a conjugate of tri-Z-spermine **71**, the final deprotection will be carried out by catalytic hydrogenolysis to remove the Z groups.

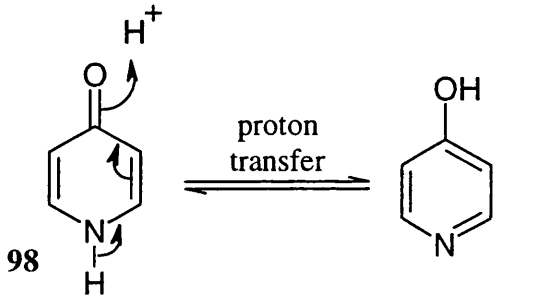
The synthesis started with 9-(10*H*)acridone **95** which was converted into 9-chloroacridine **96** by treatment with thionyl chloride in the presence of a catalytic amount of DMF (Fig. 3.2). The starting acridone **95** contains a vinylogous amide which could potentially exist as two tautomers, the favoured form being the keto rather than

the enol. This is comparable to the analogous 2- or 4-hydroxypyridine **98** compounds which also exist as the α- or γ-pyridones when in ethanolic solution, however in the vapour phase the hydroxy tautomers predominate (Fig. 3.3) (Beak *et al.*, 1976).



95

9(10*H*)acridone -
a vinylogous amide



98

solution phase vapour phase

Fig. 3.3

The reactants were heated for 30 min then the mixture was basified by pouring the reaction mixture into an ice cold solution of dilute aqueous NH_3. The product **96** was recovered by extracting with CH_2Cl_2 and, as it displayed satisfactory NMR spectroscopic data, it was used without further purification. The mass spectra of 9-chloroacridine **96** showed the expected isotope peaks associated with molecules containing ^{35}Cl and ^{37}Cl. Within 24 h of this preparation, the material was converted into 9-phenoxyacridine **97**. Sodium hydroxide was dissolved in phenol being stirred at 80 °C and then 9-chloroacridine **96** was added in one portion. After 1.5 h at this temperature the reaction mixture was poured into aq. 2M sodium hydroxide and this mixture was stirred for a further 18 h. The product **97** precipitated and was collected by filtration. After drying *in vacuo* over P_2O_5, NMR spectroscopic analyses showed the

compound to be pure and ready to be reacted as the required electrophile. It was found to be stable at 20 °C for extended periods of time (>1 year).

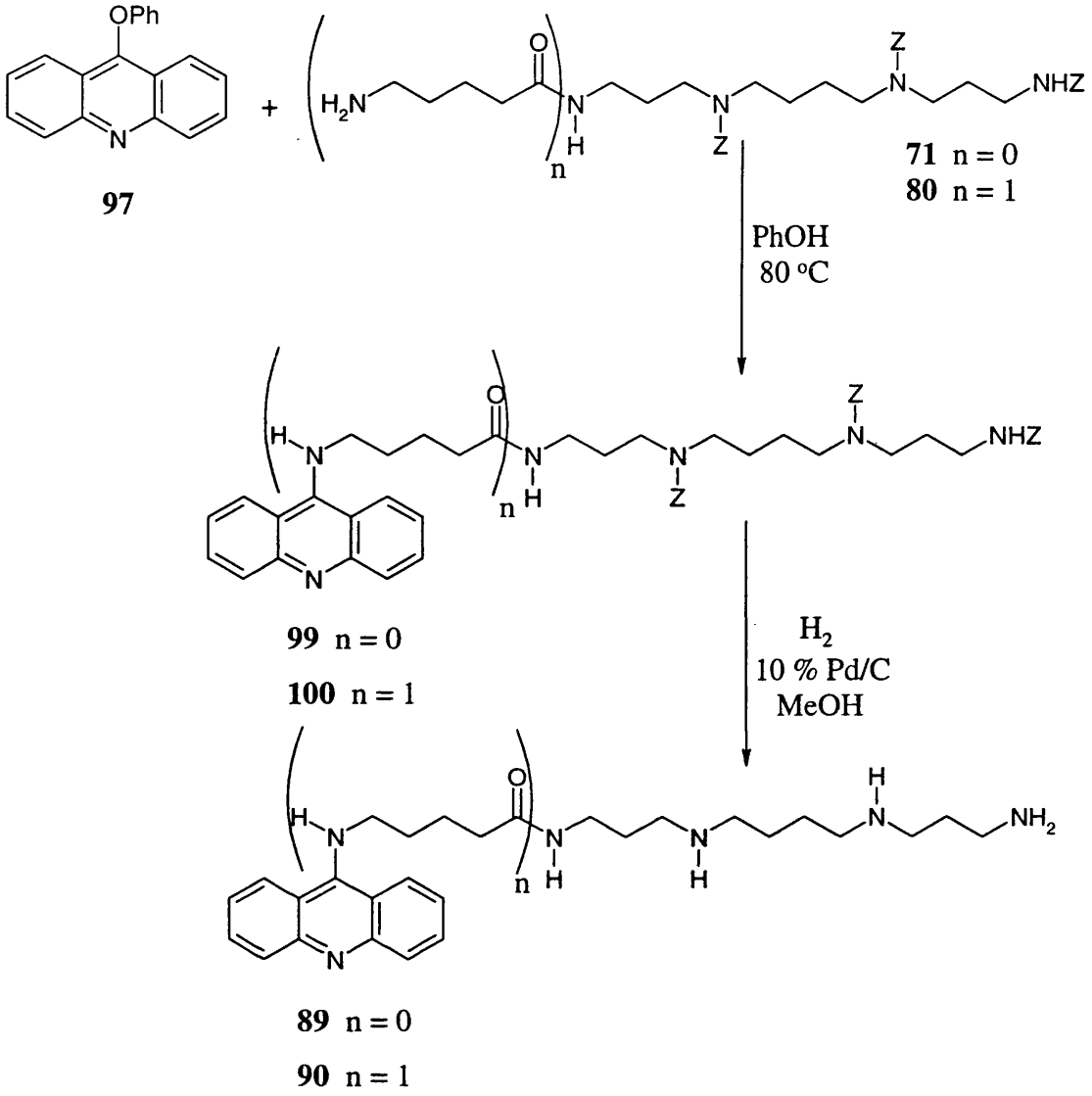


Fig. 3.4

The phenoxide **97** was displaced by the amino group in tri-Z-spermine **71** by mixing equimolar quantities in molten phenol at 80 °C (Fig. 3.4). This reaction mixture was stirred at this temperature for 18 h with TLC monitoring. After this time, there was an obvious product spot on the TLC plate which showed up strongly under UV light and was coloured bright yellow to the naked eye. The reaction mixture was diluted with CH_2Cl_2 and the resulting solution was washed with aq. 2M sodium hydroxide in order to remove the phenol (leaving group and solvent). The product was purified by flash

column chromatography on silica gel and was recovered as a bright yellow viscous oil. NMR spectroscopic analysis clearly showed that the polyamine had been conjugated to the acridine and that the protecting groups were still intact (**99**). Hydrogenolysis to remove the protecting groups followed and, after work up, TLC showed that chromatography was required to purify the product **89**.

Due to the presence of several amino groups, TLC required conc. aq. NH_3 to move the compound from the baseline. However, when flash column chromatography on silica gel was attempted using 4:2:1 CH_2Cl_2/MeOH/conc. aq. NH_3, TLC of the recovered fractions revealed only a high running spot which showed strongly under UV light and a polar polyamine spot with no UV activity, staining when heated with ninhydrin. The aq. NH_3 in the eluant was causing the product **89** to decompose on the column. Using alternative organic bases in the eluant either gave the same result or failed to move the product down the column so an alternative solid phase was sought. Neutral alumina which had been deactivated was found to be suitable. The alumina was deactivated to Brockman grade 4 by adding 10 mL of water to every 100 g of neutral alumina and shaking in a sealed container for 16 h. A gradient of eluant polarity was run through the column starting with 20 % MeOH in CH_2Cl_2 and ending at 100 %, required to prevent co-elution of by-products with the target **89** compound. The acridine-spermine conjugate **89** was finally isolated in 88 % yield as a bright yellow hygroscopic foam.

The conjugate with the 5-carbon spacer **90** was synthesised in an analogous fashion to the compound without a spacer **89**. The chain extended tri-Z-spermine **80**, as described in Chapter 2, was condensed with 9-phenoxyacridine **97** in molten phenol and the resulting conjugate **100** was isolated in 49 % yield after chromatography. Deprotection by catalytic hydrogenolysis and then chromatography on Brockman grade 4 neutral alumina yielded the target compound **90** in 79 % yield (Fig. 3.4).

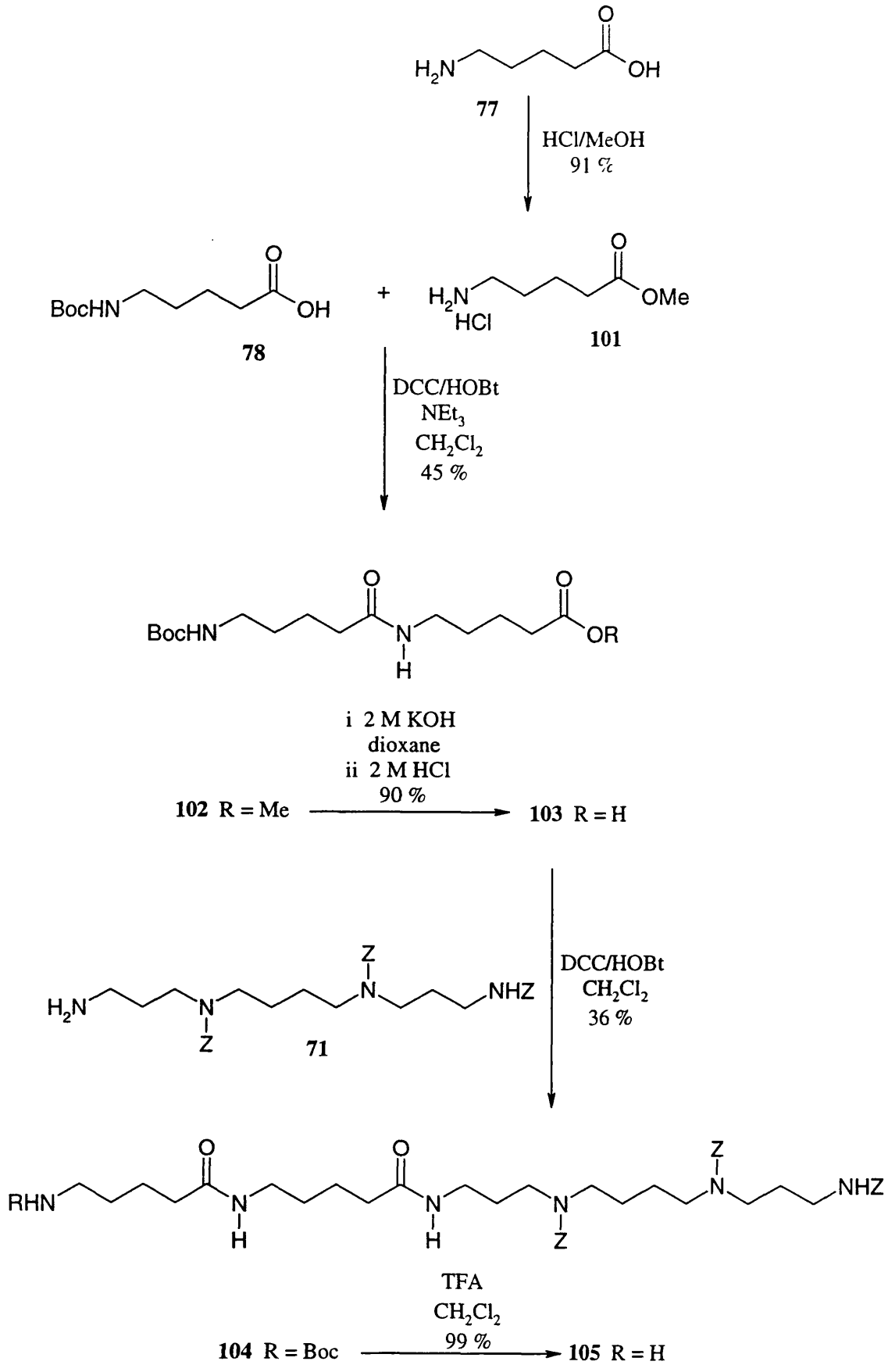

77
HCl/MeOH
91 %
78
101
DCC/HOBt
NEt3
CH2Cl2
45 %
i 2 M KOH
dioxane
ii 2 M HCl
90 %
102 R = Me
103 R = H
71
DCC/HOBt
CH2Cl2
36 %
TFA
CH2Cl2
99 %
104 R = Boc
105 R = H

Fig. 3.5

In order to synthesise the compound containing the 10-carbon spacer **91**, the spacer **103** first had to be made before being linked to tri-Z-spermine **71** (Fig. 3.5). The spacer was to be built from two 5-aminopentanoic acid **77** units. This amino acid was protected on the carboxylic acid functionality by conversion into methyl ester **101** and then it was coupled with the Boc protected 5-aminopentanoic acid **78** described previously. Ester hydrolysis would unmask the acid group ready for another amide formation step, this time with tri-Z-spermine **71**. The fully protected chain extended polyamine **104** would then be subjected to TFA mediated cleavage of the Boc group to leave it ready to be conjugated to acridine **105**.

5-Aminopentanoic acid **77** was converted smoothly into the hydrochloride of its methyl ester **101** by heating under reflux in acidified methanol. The product, a white solid, was recrystallised from methanol and was identified by the appearance of the required methyl peak at δ 3.68 ppm in its ^{1}H-NMR and by a melting point (140-141 °C) matching closely with the literature (145-146 °C, Oelofsen and Li, 1968). This compound was coupled with *N*-Boc-5-aminopentanoic acid **78** by DCC/HOBt coupling in CH_2Cl_2. NEt_3 was added to the reaction mixture to free the acid from its salt *in situ*. The coupled product **102** was isolated in 45 % yield after chromatography and was then subjected to alkaline hydrolysis with aq. KOH in dioxane. After 2 h, TLC showed that the ester had been fully cleaved and an acidic work-up yielded the desired carboxylic acid **103** in 90 % yield after chromatography.

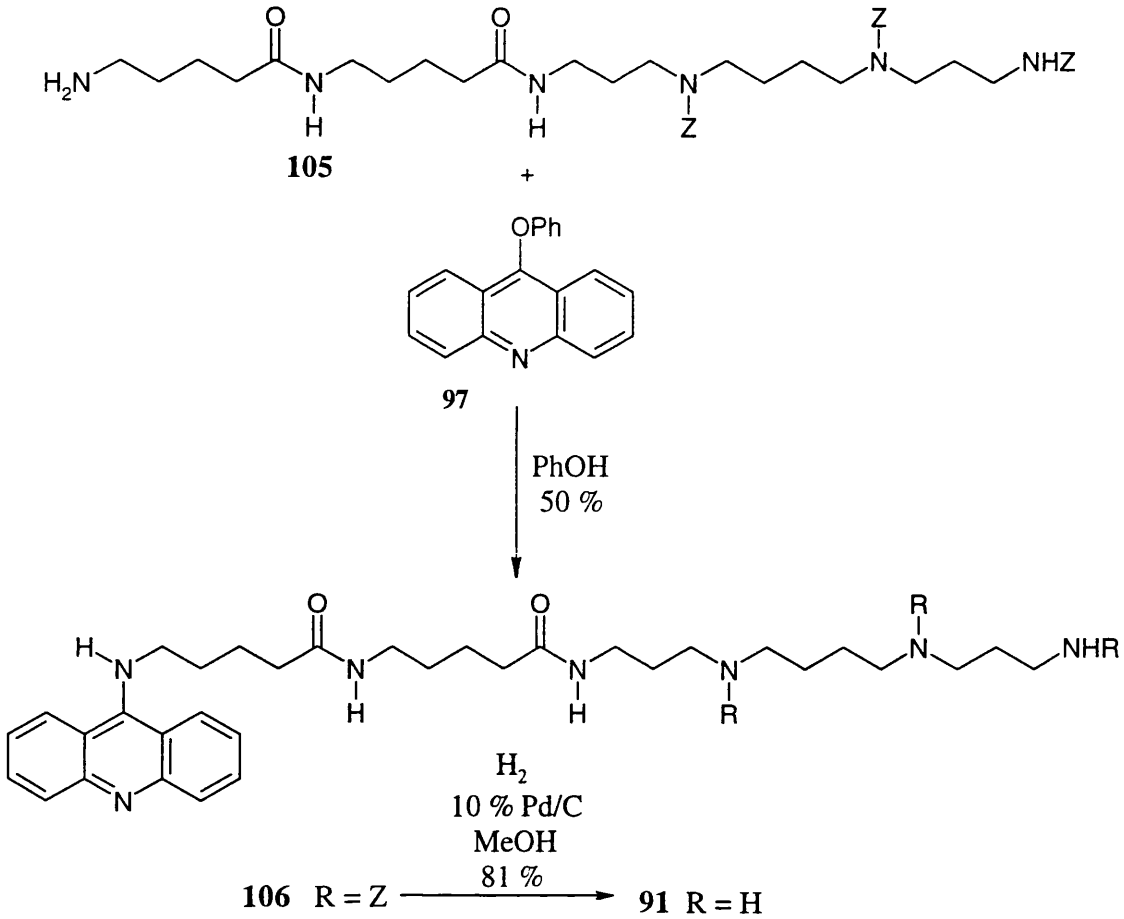
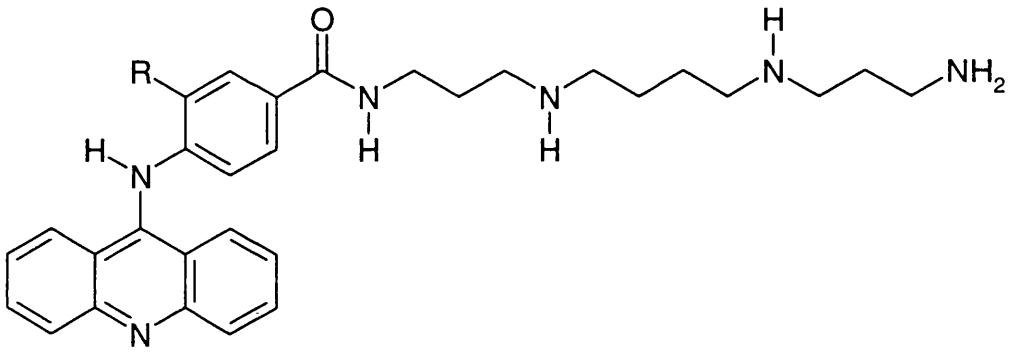


Fig. 3.6

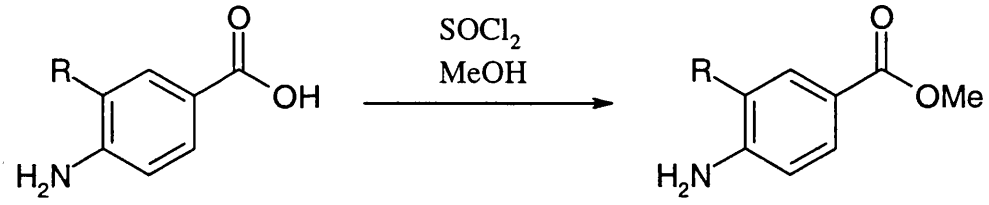
Coupling with tri-Z-spermine **71** went in 36 % yield after purification and cleavage of the Boc group yielded the mono amine **105** in quantitative yield. Coupling with 9-phenoxyacridine **97** was successfully achieved as outlined above by stirring the reactants at 80 °C in phenol yielding the protected intermediate **106** in 50 % yield. The final compound **91** was recovered after hydrogenolysis and subsequent purification on deactivated alumina in 81 % yield as a yellow hygroscopic foam (Fig. 3.6).

The compounds described so far should exhibit good affinity for DNA and display bifunctional modes of DNA interaction, but the one feature they may not have is a region which will protrude from the groove and give an area of potential interaction with DNA associated proteins, especially topoisomerases. To address this issue, two further targets **107** and **108** were identified having the aromatic pharmacophore of amsacrine **63** linked to spermine **1**.



107 R = H
108 R = OMe

One target has a 4-aminobenzoic acid derived spacer **107** between the acridine and polyamine and the other includes a methoxy group **108**, identified as being important for amsacrine structure-activity relationships, *ortho* to the amine (aniline) linkage. In the planned synthesis of these analogues, it was decided to conjugate the spacer first to the acridine rather than the spermine moiety in a fashion similar to that used in the published synthesis of amsacrine **63** (Denny, 1993). In this way, tri-protected spermine is not introduced until the penultimate stage. As preparing this protected polyamine is time consuming in terms of the chromatography required, it is more efficient to synthesise the aromatic system first, thus minimising the amount of polyamine lost through reactions not giving quantitative yields. The syntheses were started from 4-aminobenzoic acid **109** or 4-amino-3-methoxybenzoic acid **110** respectively.



109 R = H
110 R = MeO

111 R = H
112 R = MeO

Fig. 3.7

The carboxylic functionalities were initially protected in 80 % yield by treatment with thionyl chloride which had been previously reacted with methanol at 20 °C (Fig.

3.7). After 18 h stirring, neutralisation, then extraction and recrystallisation gave pure samples of the required esters **111** and **112**. Conjugation to the 9-position of acridine was brought about by nucleophilic displacement of chloride from freshly prepared 9-chloroacridine **96**. This employed the procedure described by Denny (1993) towards the synthesis of amsacrine. Previously, we had chosen phenol as the leaving group as 9-phenoxyacridine is stable at 20 °C over long periods of time (> 1 year). For these reactions using an aniline nucleophile, it was decided to follow the literature precedent and use freshly prepared 9-chloroacridine **96** for each reaction. The reactants were stirred together in anhydrous methanol and methanesulfonic acid was added to the reaction mixture. There was an immediate change in colour from yellow to deep orange-red. After 18 h, the reaction mixture was neutralised, extracted and the products were purified by flash column chromatography on silica. The ^{1}H-NMR spectrum clearly showed extra aromatic signals at δ 6.80 ppm (C$\underline{H}^{3'}$ and C$\underline{H}^{5'}$) and 7.82 ppm (C$\underline{H}^{2'}$ and C$\underline{H}^{6'}$) due to the incorporation of the six-membered ring to the acridine. These compounds are an orange colour, whilst the starting acridine compound is yellow. The change in the system leads to increased conjugation in the aromatic chromophore and a shift of the absorption maxima to longer wavelengths. It is also noteworthy that the nucleophilic nitrogen is an aniline, and contains a *para*-substituted electron withdrawing group, these groups acting in a complementary manner to increase further the red-shift (bathochromic). Furthermore, the nucleophilicity of this moiety will be significantly reduced by the electron withdrawing group, and unsubstituted anilines are poor nucleophiles. Therefore, the successful synthesis of the desired targets **113** and **114** in good overall yield and purity is an important goal towards the required final target molecules.

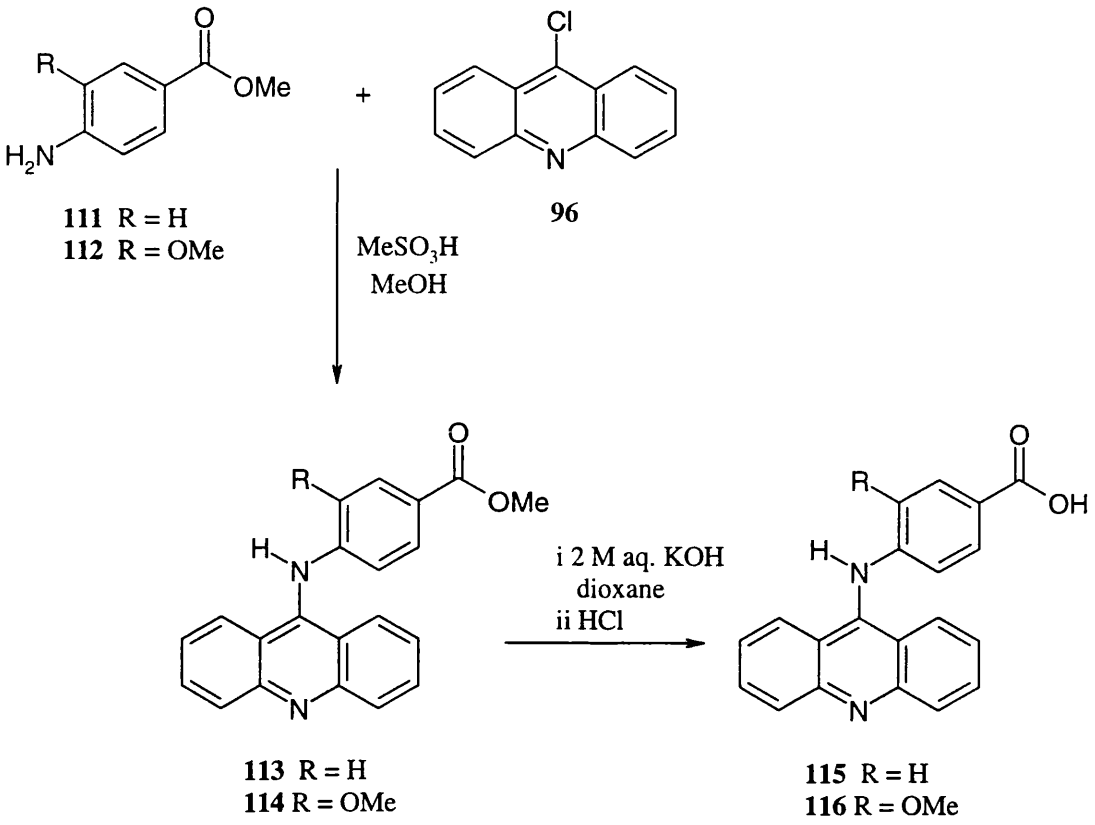

R
OMe
H_2N
+
Cl
N
111 R = H
112 R = OMe
96
$MeSO_3H$
MeOH
R
OMe
H
N
N
i 2 M aq. KOH
dioxane
ii HCl
R
OH
H
N
N
113 R = H
114 R = OMe
115 R = H
116 R = OMe

Fig. 3.8

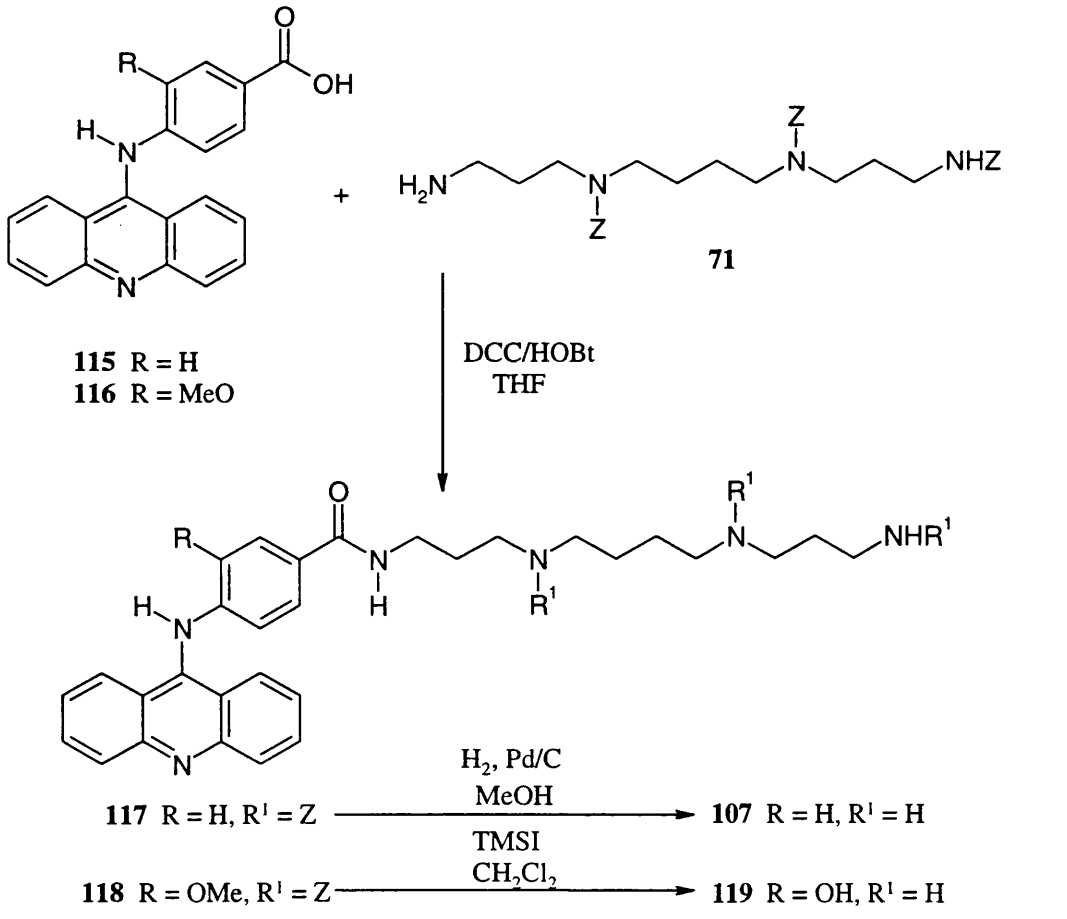

R
OH
H
N
N
+
H_2N
N
Z
N
Z
NHZ
71
115 R = H
116 R = MeO
DCC/HOBt
THF
R
N
H
N
R^1
N
R^1
NHR^1
H_2, Pd/C
MeOH
117 R = H, R^1 = Z
107 R = H, R^1 = H
TMSI
CH_2Cl_2
118 R = OMe, R^1 = Z
119 R = OH, R^1 = H

Fig. 3.9

The esters **113** and **114** were saponified by the action of aq. KOH in dioxane and once protonated and purified, the free acids **115** and **116** were linked to tri-Z-spermine **71** by DCC-HOBt coupling (Fig. 3.9). Initial attempts at coupling were carried out over 24 h, but after this time significant amounts of the intermediate formed between DCC and the acid were isolated. This intermediate must have significant stability as it survived chromatography. After 48 h of reaction, modest amounts of the desired amides **117** and **118** could be isolated.

The final deprotection of the conjugates **117** and **118** was attempted, as previously described, by catalytic hydrogenolysis. The analogue without the methoxy group **107** on the anilino ring deprotected after 24 h and was isolated in 61 % yield after chromatography as an orange hygroscopic foam. When this procedure was applied to methoxy analogue **118** there was no recovery of the desired product **108** and there was considerable breakdown of the starting material as indicated by TLC. As there were many product spots, no attempt was made to identify them. Attempts were also made at hydrogenation with Pearlman's catalyst ($Pd(OH)_2/C$) both using MeOH and AcOH as solvents, but only similar disappointing results were achieved. An alternative method of Z group removal is by the action of trimethylsilyl iodide (TMSI) and this was attempted but, as predicted, this method also bought about *O*-demethylation. This novel analogue **119** was purified, characterised and was tested with the other compounds.

As the removal of Z groups was proving to be problematic, it was decided to use a slightly less rigorous protection strategy to synthesise the required 3'-methoxy analogue **108** (Fig. 3.10). The tetracyclic acid **116** was coupled to N^1-Boc-spermine **69** using DCC and catalytic HOBt. The greater nucleophilicity of the primary amine over the secondary amines was relied upon to yield the required di-acylated polyamine **120**. After work-up and column chromatography, the desired compound **120** was isolated in

37 % yield. TFA in CH_2Cl_2 was used to remove the Boc group and successfully yielded the desired target compound **108** as its polytrifluoroacetate salt. TLC and analytical reverse-phase HPLC showed the compound to be pure and requiring no further chromatography.

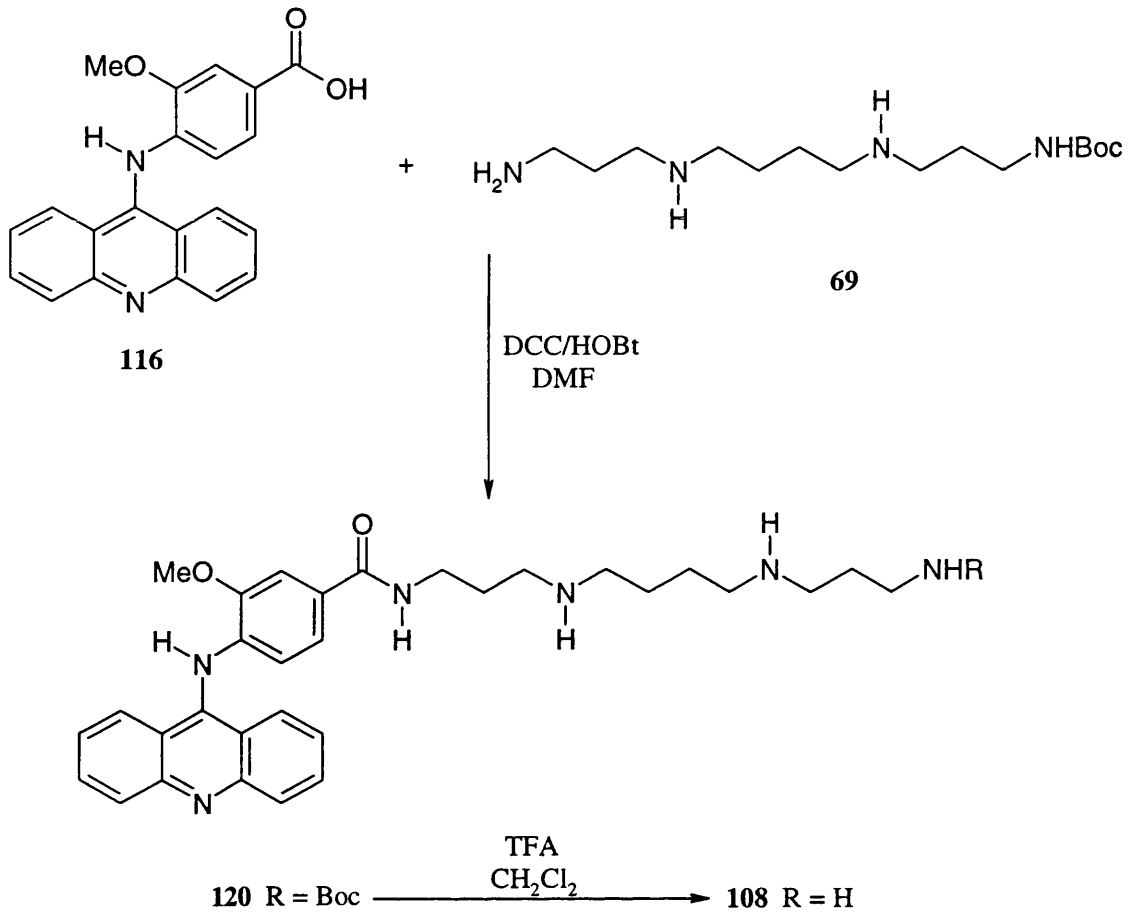


Fig. 3.10

The 9-aminoacridine conjugates were assayed for cytotoxic activity against B16 murine melanoma cells by M. A. Qarawi in the MTT assay as described above (Chapter 2). The results of these assays are shown in Table 3.1.

Table 3.1 Results of the MTT assay of 9-aminoacridines

Compound	Mean EC_{50} (M)	SE (±)
Acrid-3.4.3 **89**	$1.0x10^{-6}$	$5.6x10^{-7}$
Acrid-4-CO-3.4.3 **90**	$2.7x10^{-7}$	$1.2x10^{-8}$
Acrid-4-CO-4-CO-3.4.3 **91**	$3.0x10^{-5}$	$2.0x10^{-5}$
Acrid-aniline-CO-3.4.3 **107**	$9.2x10^{-6}$	$2.3x10^{-6}$
Acrid-(HO-aniline)-CO-3.4.3 **119**	$9.0x10^{-5}$	$3.0x10^{-5}$
Acrid-(MeO-aniline)-CO-3.4.3 **108**	$4.9x10^{-6}$	$5.7x10^{-7}$

Table 3.1 shows the concentration of each compound (EC_{50}) required to kill 50 % of the cells exposed to it.

The 9-aminoacridine-spermine conjugate **89** showed improved cytotoxic activity over the 9-carbonyl analogue. This was predicted as certain 9-aminoacridines have been shown to have notable antitumour activity, but amidoacridines do not. The addition of a 5-carbon spacer increased the activity and gave the most potent compound in this study. When the spacing between the intercalator and polyamine was increased to ten there was a drop in cytotoxic activity. The compounds having the amsacrine aromatic core displayed activity of about the same order as the 9-amidoacridines in this assay. The most cytotoxic compound was the 3'-methoxy analogue **108** closely followed by the unsubstituted (3-hydrido) **107**. There was a drop off of about an order of magnitude of activity in the hydroxy analogue **119**.

These 9-aminoacridine-polyamine conjugates are promising leads as we have achieved over an order of magnitude greater cytotoxic activity than the 9-amido compounds previously described (Chapter 2). These compounds offer ideal frameworks for the investigation of both DNA binding and cytotoxic modes of action, particularly their interaction with topoisomerases and other DNA associated proteins. We have demonstrated that polyaromatic-polyamine conjugates with multiple modes of DNA binding have enhanced cytotoxic activity over either of their components which have only single modes of DNA interaction.

Chapter 4

Solid Phase Approaches to the Synthesis of Polyamines and Polyamine Amides

Solid Phase Approaches to the Synthesis of Polyamines and Polyamine Amides

Despite the well developed repertoire of protecting group manipulations described above (Chapters 2 and 3) and in the literature, (for reviews, see: Greene and Wuts, 1991; Kocienski, 1994; Schelhaas and Waldmann, 1996), the synthesis of polyamines, especially unsymmetrically substituted polyamines, can still be time consuming and at times low yielding. If the polyamine required is not available naturally, it must be built up in a stepwise fashion. At all times protecting groups must be carefully chosen in order to allow orthogonal introduction and deprotection for access to specific amines for further conjugation. Generally, a chromatographic purification is required after each synthetic step to remove undesirable by-products (e.g. acylation or alkylation at unwanted positions) and unreacted starting materials. A potentially more rapid approach to the synthesis of polyamines and polyamine conjugates is by the use of solid phase organic synthesis (SPOS) (Nash *et al.*, 1996; Byk *et al.*, 1997; Kellam *et al.*, 1997; Tomasi *et al.*, 1998; Page *et al.*, 1998). A strategy can be envisaged whereby the terminal primary amine of a suitable protected diamine or polyamine is attached to a resin and then the free primary amine is reacted sequentially either to lengthen the polyamine chain or for the addition of other moieties (e.g. acridine) on the way to conjugates of biological interest (Fig. 4.1).

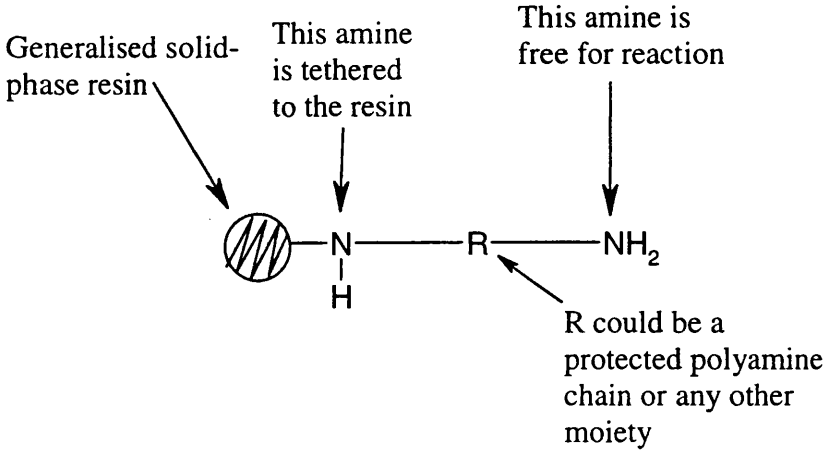
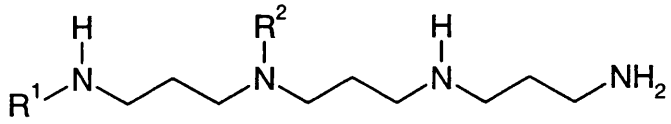


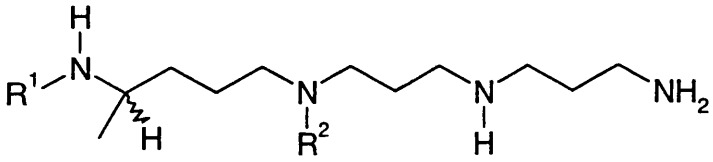
Fig. 4.1

The major advantages of SPOS over more traditional synthesis are that the intermediate purification steps are not necessary and yields are higher. Each step in the synthetic sequence is usually driven to total completion by the use of large excesses of reagents, a situation which is not always possible in solution phase synthesis. At the end of the reaction time, the resin is filtered and the excess of reagents is washed away by repeated passage of solvent over the resin. When all of the reactions are completed, there is a cleavage step and, if necessary, a single chromatographic purification to yield the desired product. A further advantage of SPOS is the possibility of using combinatorial approaches to build quickly libraries of compounds by splitting the resin, reacting each portion with a different reagent, recombining then splitting and reacting again. The major disadvantage associated with SPOS is that the reactions employed must give only one product since, after multiple steps, reactions with side products will quickly multiply the number of compounds on the resin leading to poor yields and difficult separations after cleavage. For the same reasons, all reactions must be quantitative or as close to 100 % reaction as possible. There are also a number of practical considerations which must be taken into account when conducting SPOS. Solvent systems must be chosen carefully to ensure that the resin swells efficiently. If the reaction solvent does not allow the resin to swell sufficiently, the reagents will not be able to penetrate to all of the bound substrate and there will be incomplete reaction. There is also the issue of how the courses of the reactions are monitored. The use of TLC is not normally convenient as it requires the cleavage of a small quantity of the substrate from the resin which is time consuming and depletes the quantity of resin over several successive steps. This problem has led to the development of specific techniques for reaction monitoring e.g. FTIR and gel-phase ^{13}C-NMR (Look *et al.*, 1994).

Having synthesied spermine amide conjugates with acridine by solution phase techniques, six targets **121-126** were designed having polyamines with different configurations of methylene groups acylated with acridine on either a primary or a secondary amine. These compounds were to be synthesised by SPOS methodology from a common starting material, the mono-protected triamine N^1-Boc-N^1-(3-aminopropyl)-1,3-diaminopropane.

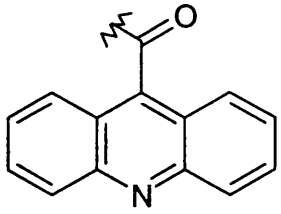


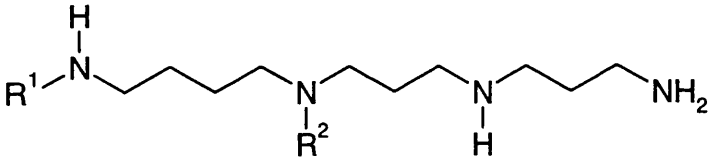
121 R^1 = Acr, R^2 = H
122 R^1 = H, R^2 = Acr



123 R^1 = Acr, R^2 = H
124 R^1 = H, R^2 = Acr

Acr =





125 R^1 = Acr, R^2 = H
126 R^1 = H, R^2 = Acr

The solid support chosen for this work was Wang resin (*para*-benzyloxybenzylalcohol polystyrene) (Wang, 1973; Lui *et al.*, 1981; Sieber, 1987) which has 0.65 mmol of hydroxyl groups per g of resin. The benzylic hydroxyl is reacted with 4-nitrophenyl chloroformate which is then reacted with the amine to be

linked to the polystyrene (Fig. 4.2). The linkage thus formed is a carbamate so the amine is protected and will not react further during the SPOS synthetic manipulations (Dixit and Leznof, 1977). This carbamate is cleaved under acidic conditions which rules out any acidic reagents in the synthetic scheme, but it also means that as the polyamine chain is lengthened, amines which need protection can be masked by Boc groups. At the end of the synthesis, exposing the resin to TFA will cleave both the resin linkage and the Boc protecting groups in one step.

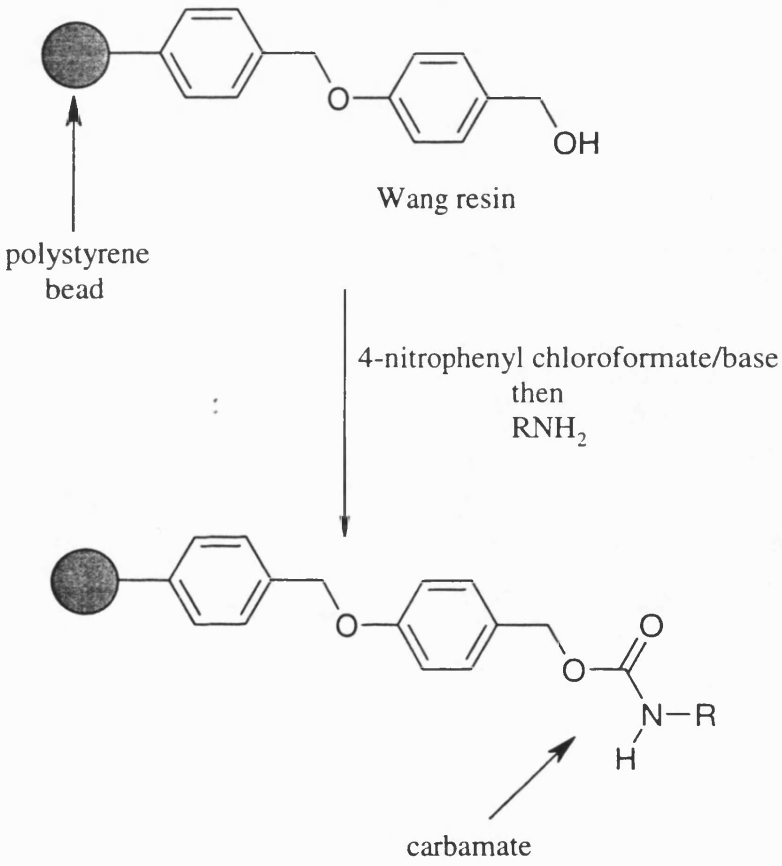


Fig. 4.2

Fig. 4.3

Fig. 4.4

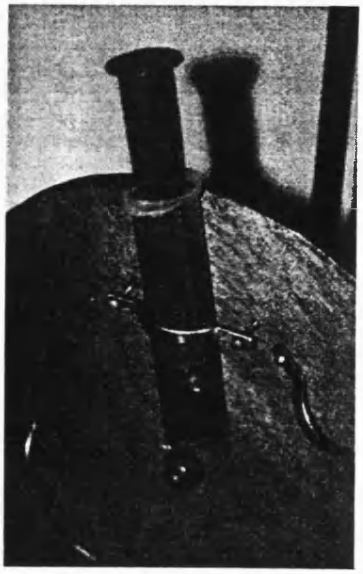
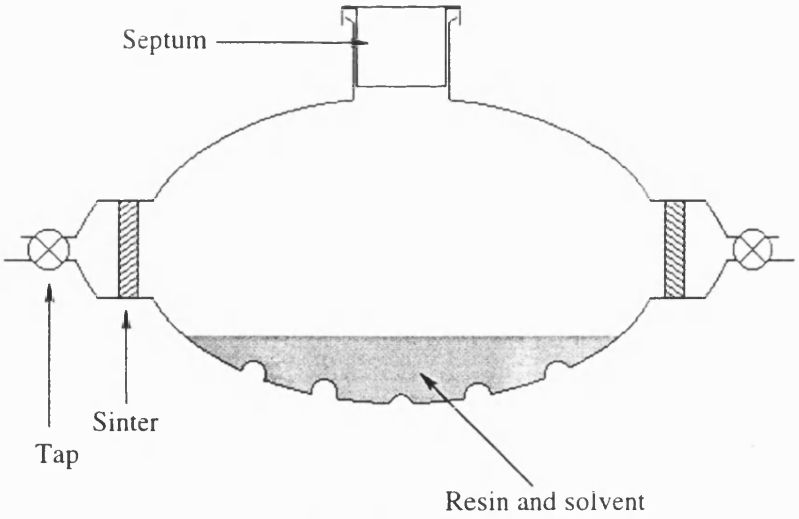


Fig. 4.5



Reactor is rocked on its side whilst the reaction is in progress

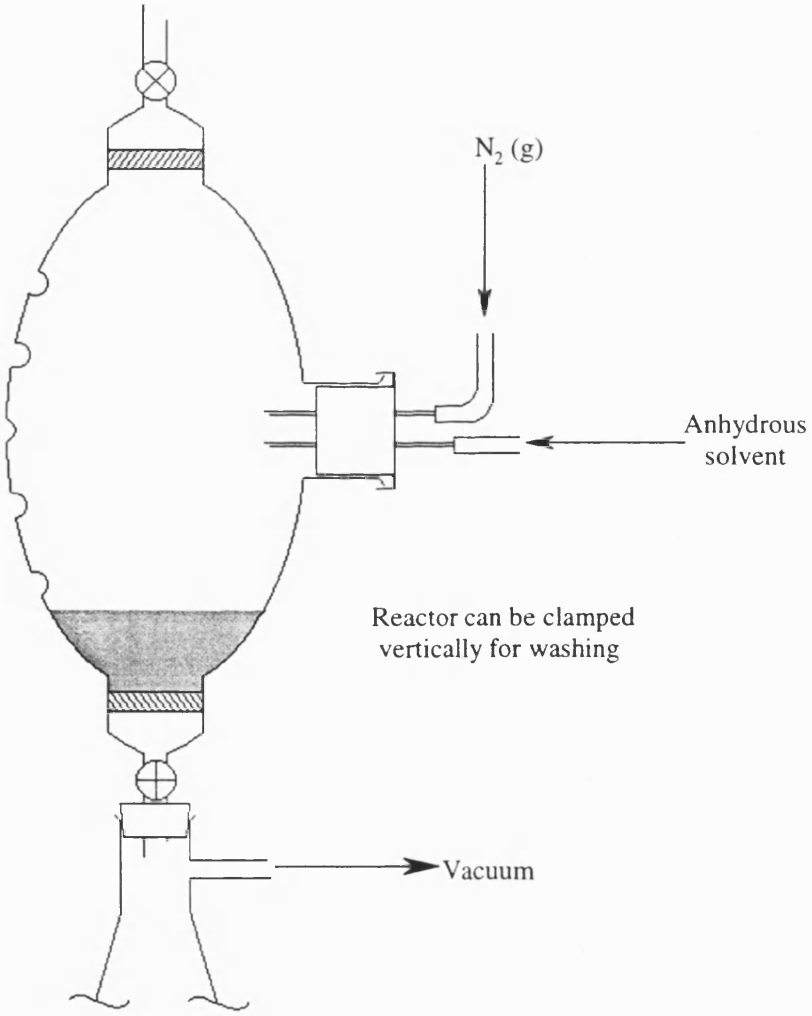
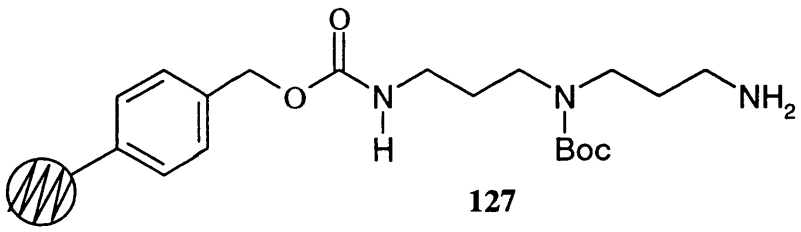


Fig. 4.6

Towards the synthesis of the target compounds **121-126**, Wang resin was reacted with 10 equiv. of both 4-nitrophenyl chloroformate and *N*-methylmorpholine. Due to the nature of the resin, reactions cannot be stirred in a conventional mechanical fashion as the beads are broken up and destroyed, so the reaction vessel is constantly rocked or agitated in order to ensure even distribution of reagents. The reaction was done on 5 g of resin in a glass reactor with three necks, one sealed with a septum and two with sinters and taps (Fig. 4.6, for a photo see Fig. 4.3). This arrangement allowed the introduction (*via* a cannula) and removal of anhydrous solvents allowing the activated product to be washed in an inert atmosphere. The reactor had a dimpled base which disperses reagent amongst the resin and it was coated with a layer of silicon (sigmacote) before introduction of the resin to prevent the resin from sticking to the glass.



127

After thorough washing of the resin with a range of anhydrous solvents, the resin was reacted with half quantities of the same reagents for a reduced time to ensure that the reaction was complete. After washing and drying the resin (40 °C over P_2O_5) it was reacted with N^1-Boc-N^1-(3-aminopropyl)-1,3-diaminopropane **181** (see Chapter 5 for synthesis) in CH_2Cl_2. As the reaction progressed and the amine displaced the 4-nitrophenol leaving group, the reaction mixture turned a bright yellow colour. After 16 h the resin **127** was washed, this time without the need for anhydrous conditions. In order to remove the final traces of 4-nitrophenol, it was necessary to agitate the resin for an extended period of time (typically 18 h) in THF-H_2O (1:1) as part of the washing

process. In order to monitor the success of the reaction, a small sample (~15 mg) was taken and ground up with solid, anhydrous KBr. The absorbance of this mixture was measured with an FTIR instrument and the appearance of a broad band at 1680-1700 cm^{-1} was clearly visible indicating the presence of the carbamate functional group.

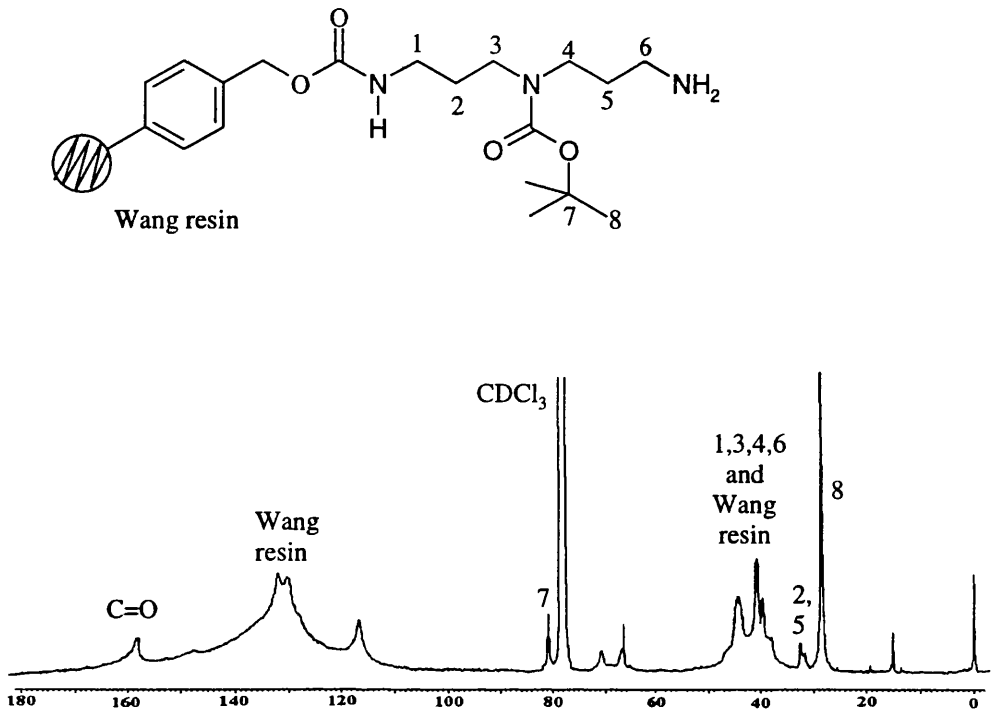


Fig. 4.7

The completion of the reaction was also verified by measuring the ^{13}C-NMR spectrum of the resin as a gel in $CDCl_3$ (Fig. 4.7).

Before extending the polyamine, some trial reactions were carried out to investigate the best method for introducing the acridine moiety. The initial idea was to use acridine-9-acyl chloride **128**. This was prepared from the carboxylic acid **73** which was reacted with thionyl chloride in the presence of a catalytic amount of DMF (Fig. 4.8). Several attempts were made to acylate the free amine on the resin bound triamine **127** with this acid chloride. Reactions were tried using *N*-methylmorpholine or NEt_3 as a base (Fig. 4.9).

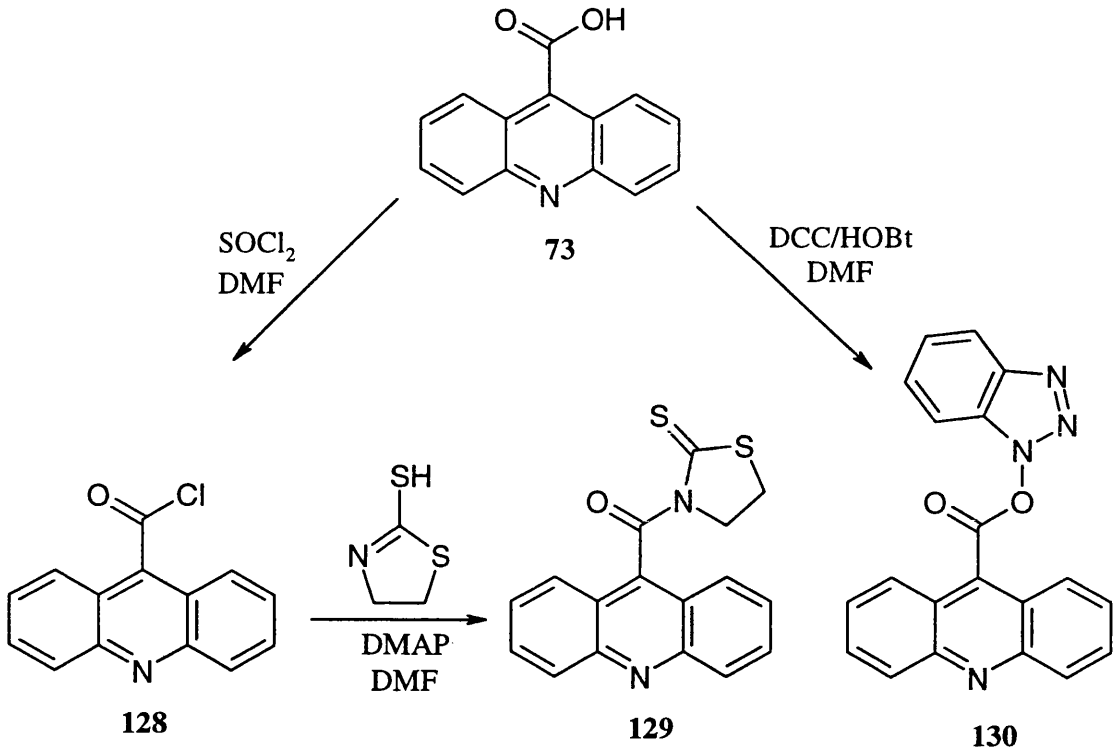


Fig. 4.8

Unfortunately, it is not possible to monitor the progress of the reaction by FTIR as the resin masks the regions where the new IR bands appear. In each case, the resin was treated with TFA in CH_2Cl_2 to cleave the product and, in each case, only unreacted polyamine was isolated as identified by TLC. Two further methods of activating the acridine-9-carboxylic acid **73** were tried (Fig. 4.8). The first was with 2-mercaptothiazoline (Nagao *et al.*, 1980 and 1984). This heterocycle was reacted with a portion of the acid chloride in the presence of triethylamine and a trace of DMAP **92**. The reaction was monitored by TLC until all of the acid chloride had been consumed then the resulting solution **129** was used to treat the resin **127**. This method also failed to yield any acylated product after cleavage. The final method of acylation to be tried was by the use of HOBt activation. Acridine-9-carboxylic acid **73** was reacted with DCC and a stoichiometric amount of HOBt in DMF. The resulting solution of activated acid **130** was used to acylate the resin **127**. This time, after TFA mediated cleavage of resin **131**, there was an obvious polyamine-chromophore conjugate in the residue (Fig.

4.9). A flash column was necessary to separate this product from unreacted triamine, that may have been cross-linked on the resin. The isolated product showed ^{1}H- and ^{13}C-NMR spectra consistent with the terminally acylated triamine **132**.

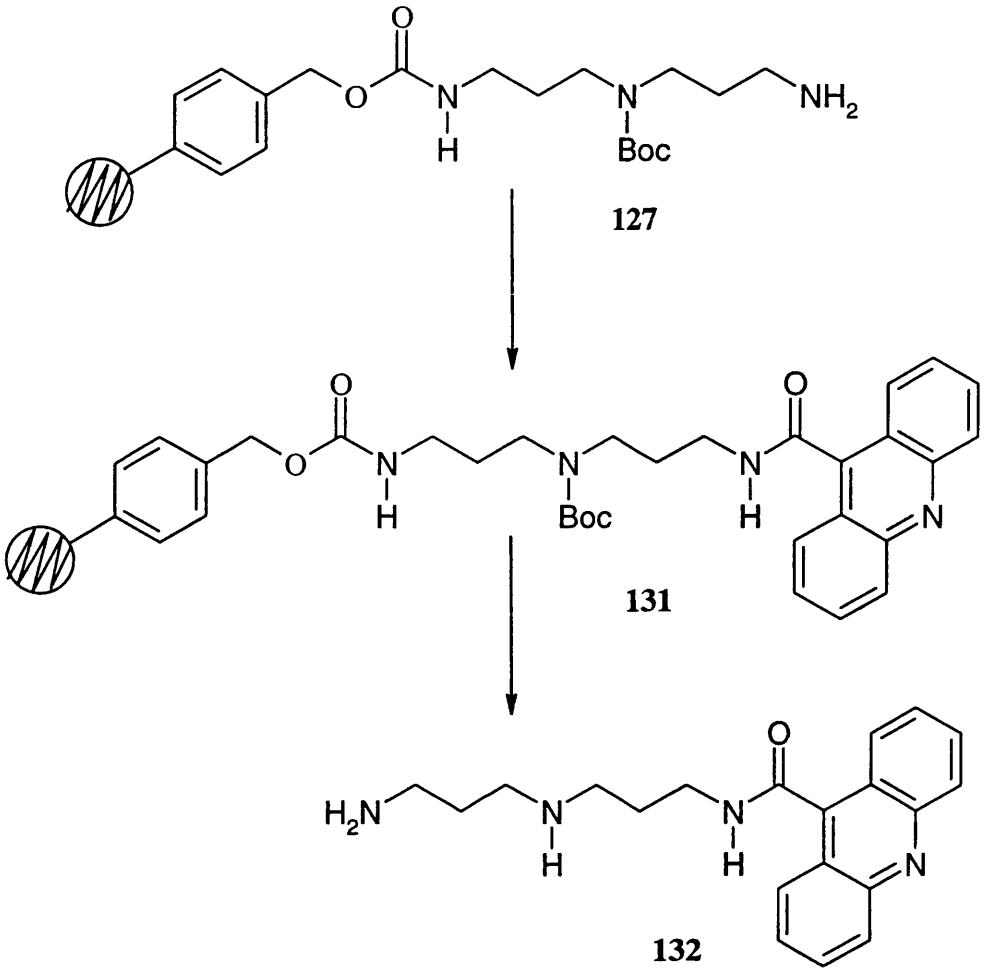


Fig. 4.9

In order to extend the polyamine chain on the resin, reductive amination reactions were proposed. The amino position to be chain extended is exposed to an *N*-protected amino-aldehyde or *N*-protected amino-ketone in the presence of a reducing agent. An imine is formed between the resin bound amine and the carbonyl, then reduction yields the secondary amines from the aldehyde or ketone (Fig. 4.10).

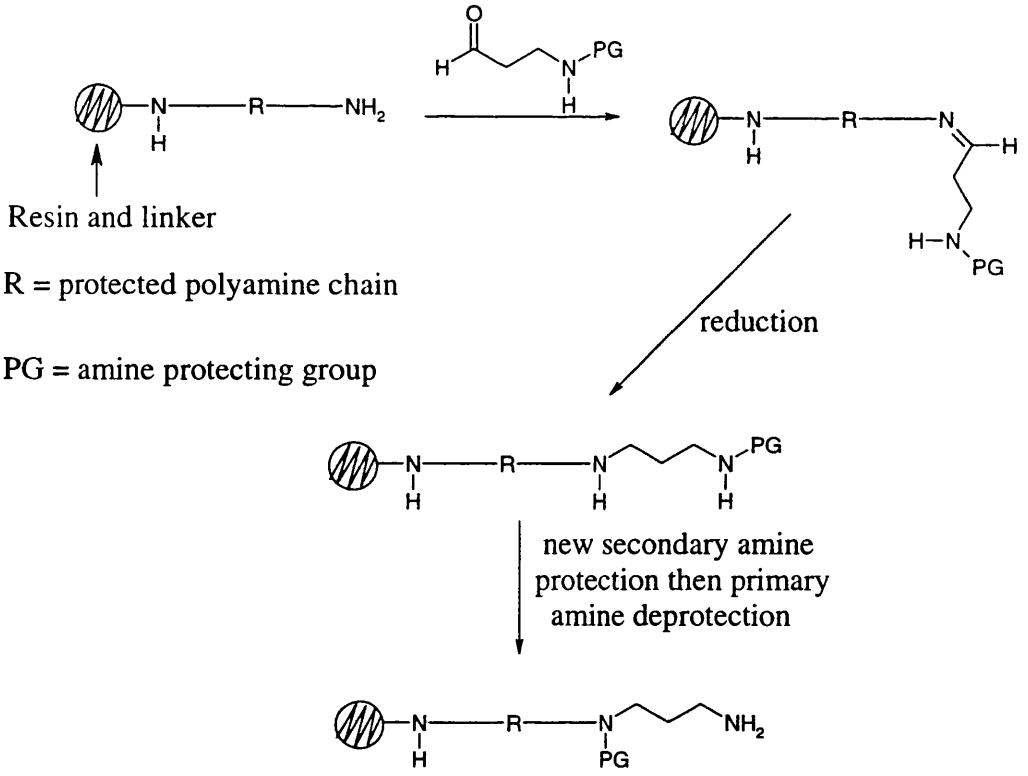


Fig. 4.10

The protecting group on the new terminal amine can then be removed generating a new site for either chain extension by a further reductive amination, or alternatively for acylation.

Due to the acid labile nature of the carbamate linker to the resin, the protecting group on the amino-aldehyde/ketone has to be chosen so that it can be cleaved in a non-acidic fashion. The Z group which was used as a protecting group orthogonally to Boc in Chapters 2 and 3 is not suitable for SPOS as the typical method of cleavage is by catalytic hydrogenation. The catalyst is a Pd species adsorbed on C and there is no obvious way of separating the solid catalyst from the resin after reaction. In this work, the use of the azido and pent-4-enoyl (Pnt) groups were investigated. The azido group can be reduced by action of PPh_3 to give an amino group (Carboni *et al.*, 1993). The Pnt group has not previously found use in the SPOS synthesis of polyamines. Its use was reported initially by Fraser-Reid and co-workers in solution-phase towards the synthesis of β-glucosaminides (Madsen *et al.*, 1995). The Pnt group has also found use in the

synthesis of oligonucleotides both in solution and solid phase syntheses (Radhakrishnan *et al.*, 1995 and 1997). It is cleaved by action of I_2 , usually in a 1:1 THF/H_2O solvent mix through a five membered cyclic intermediate giving the free amine after hydrolysis, Fig. 4.11.

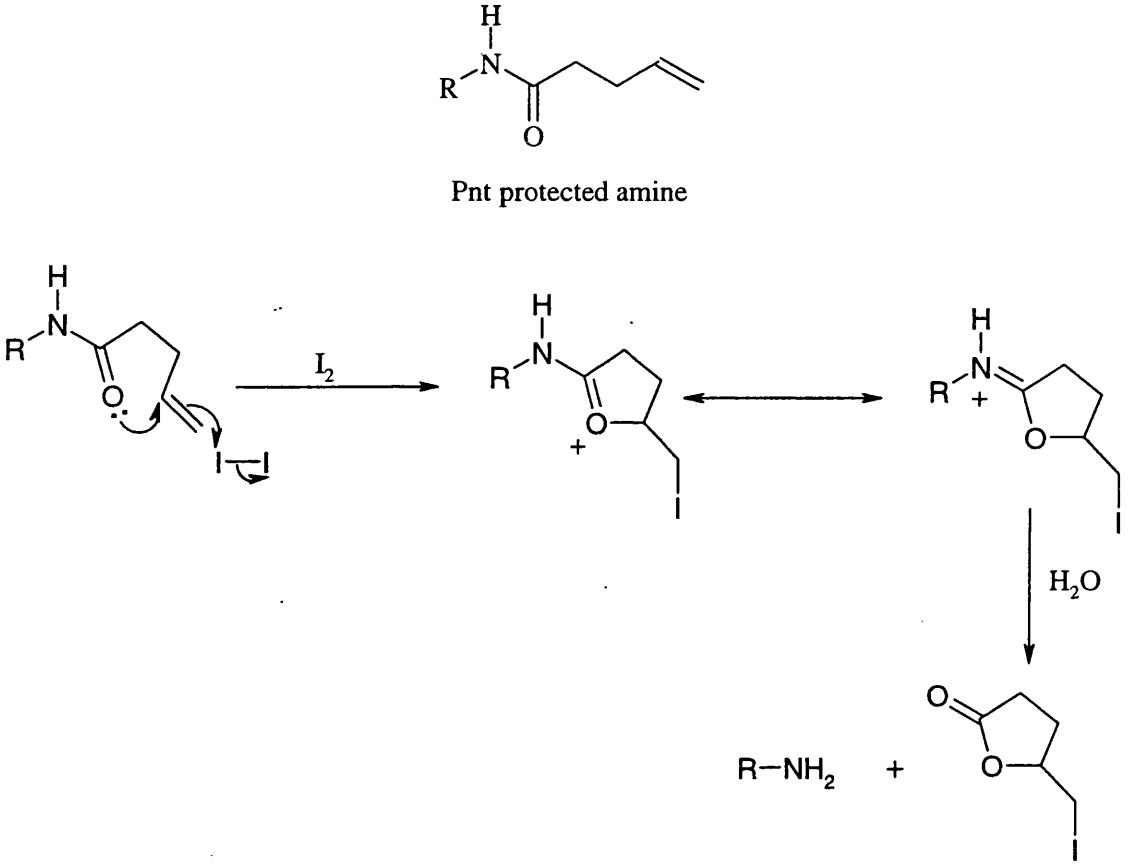
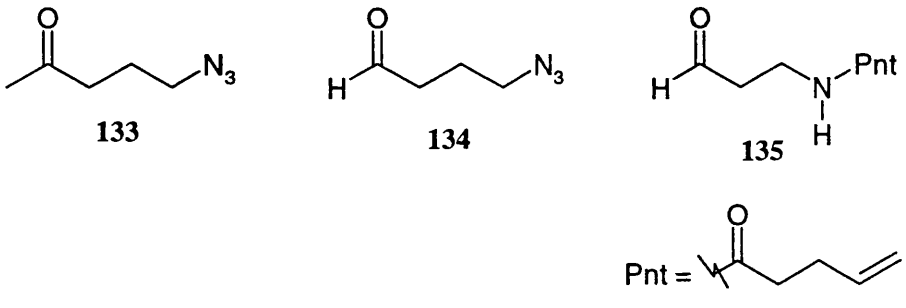


Fig. 4.11

Three carbonyl compounds were chosen for reductive amination reactions, 5-azidopentan-2-one **133**, 4-azidobutanal **134** and 3-(Pnt-amino)propanal **135**.



The reducing agent chosen to investigate these reactions was borane-pyridine complex (BAP). There was some literature precedent for the use of BAP, but this was to make tertiary rather than secondary amines (Kahn *et al.*, 1996).

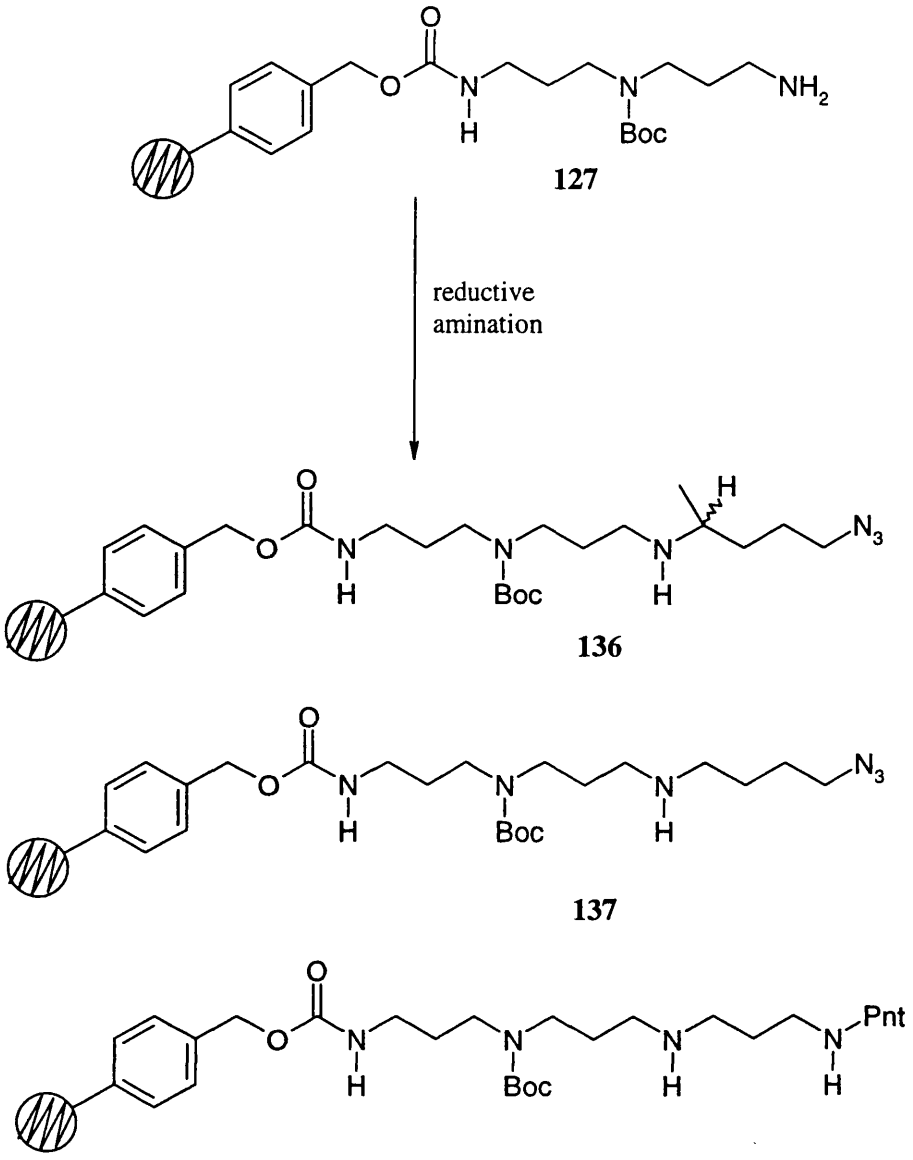


Fig. 4.12

Each reductive amination was carried out with 10 equiv. of aldehyde/ketone and the same amount of BAP (Fig. 4.12). In the case of the azido containing compounds **133** and **134**, the progress of the reaction could be monitored by the appearance of an azido band, located at 2090 cm^{-1} in the IR. The spectrum for the reaction with the Pnt protected amine **135** showed no change as the resin peaks masked any new absorbances. These reactions were all on 2 g of resin and of 48 h duration, carried out under argon

with agitation. The quantity of resin meant that it was most convenient to perform the reactions in a 50 mL round bottom flask. (for a photo see Fig. 4.4) The flask was pre-treated with sigmacote before introduction of the resin to ensure it did not stick to the glass. After the reaction time, each resin was thoroughly washed and dried and was then split into two portions. One half was reacted with a tenfold excess of Boc anhydride to protect the newly formed secondary amine (Fig. 4.13).

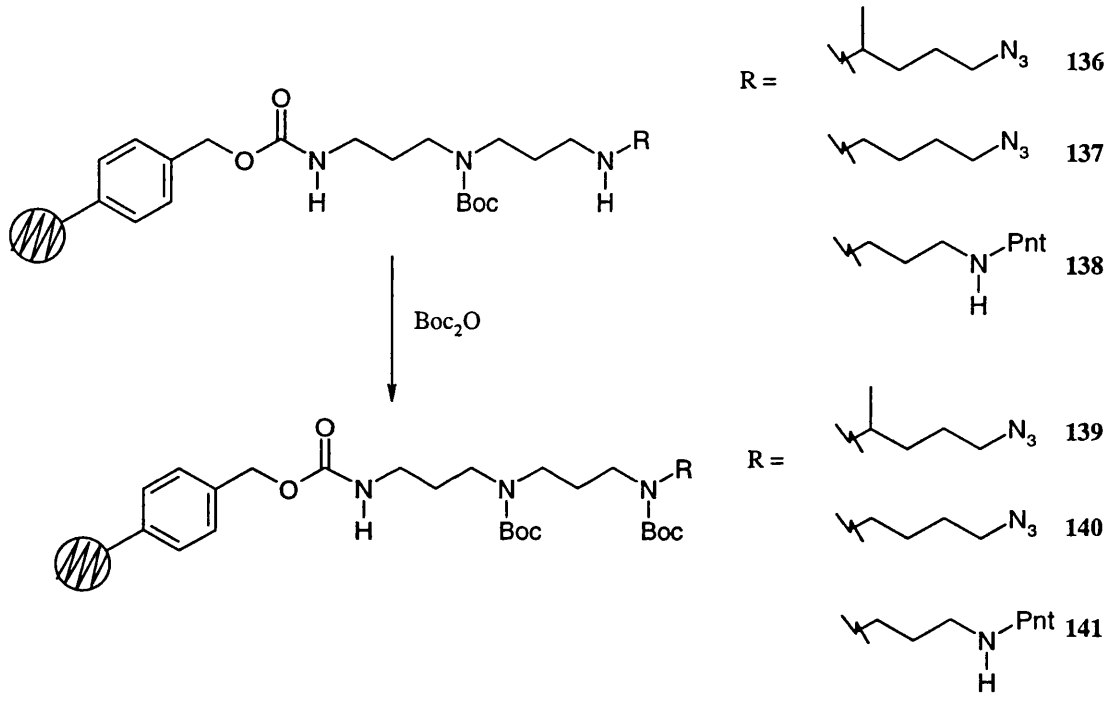


Fig. 4.13

As the quantities of resin now being used were around 1 g per reaction it was unnecessary to use a glass reactor. A convenient alternative is a plastic, 20 mL syringe with a filter at the end (Fig. 4.14, for a photo see Fig. 4.5). The resin is placed into the syringe and the plunger replaced. Solvent can then be sucked up and, more importantly, removed by simply drawing up or pushing down the plunger, the filter preventing the resin from leaving the syringe. In this way, reagents can quickly be introduced then the end of the syringe is fitted with a cap. Simple rotation of the syringe ensures dispersion of reagent during reaction and the washing at the end is quick, efficient and easy.

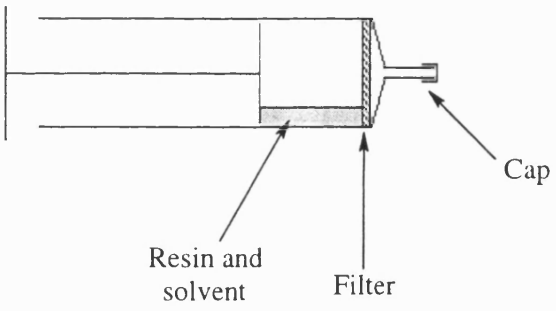


Fig. 4.14

After the Boc protection step, the two resins bearing the azido functionalities (**139** and **140**) were reacted with PPh_3 in THF with a small amount of H_2O in order to reduce the azides to amines (Fig. 4.15). The course of the reaction was easily followed by monitoring the disappearance of the azide peak in the IR characteristically at 2090 cm^{-1} (Fig. 4.16).

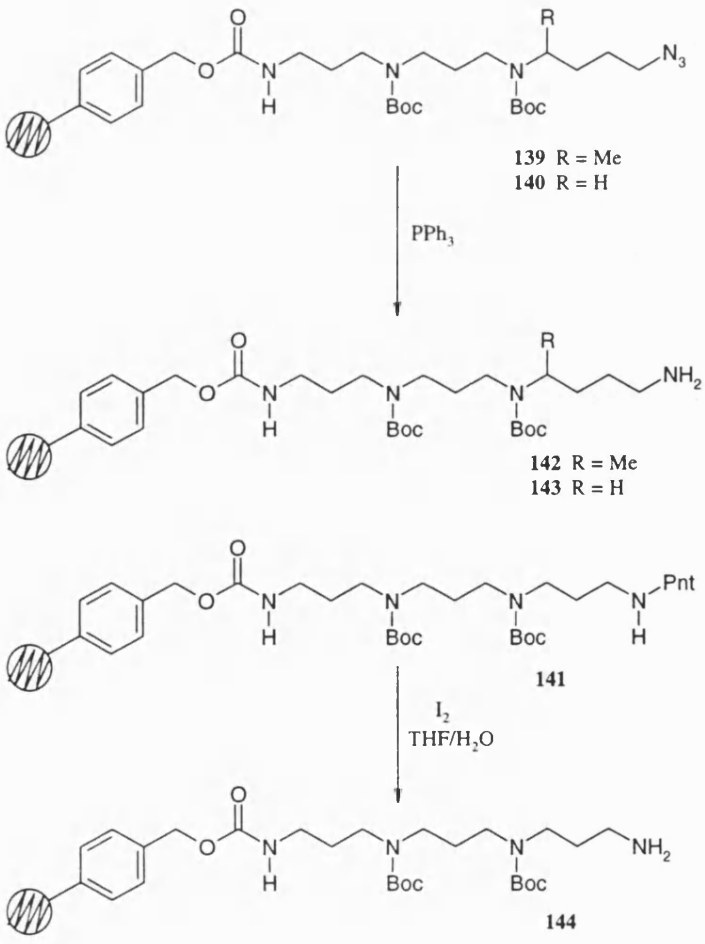


Fig. 4.15

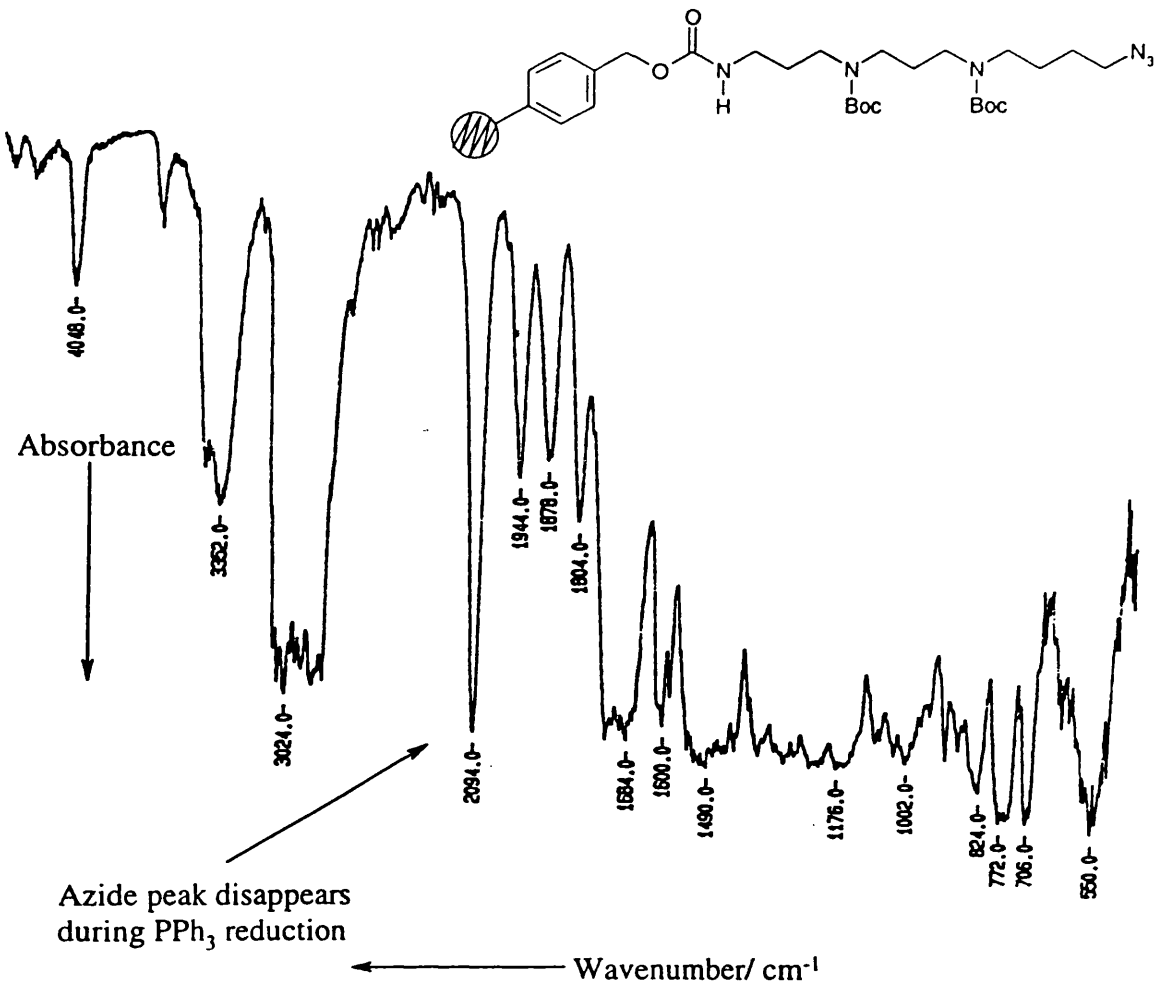


Fig. 4.16

Two days were required for complete reaction and after washing and drying then each resin was acylated with a solution of HOBt activated acridine-9-carboxylic acid **130** in DMF. These reactions were performed over 19 h and during this period the acylating reagent was replaced once (Fig. 4.17).

The resin bearing the Pnt protected amine **141** was reacted with I_2 in a 1:1 mixture of THF and H_2O. Ten equiv. of I_2 were used and the reaction was run for 18 h. When the resin **144** was washed after reaction, as well as the usual range of solvents, a saturated solution of aq. $Na_2S_2O_3$ was used to remove traces of I_2. This resin was then acylated in the same way as the previous two with HOBt activated acridine-9-carboxylic acid **130** (Fig. 4.17).

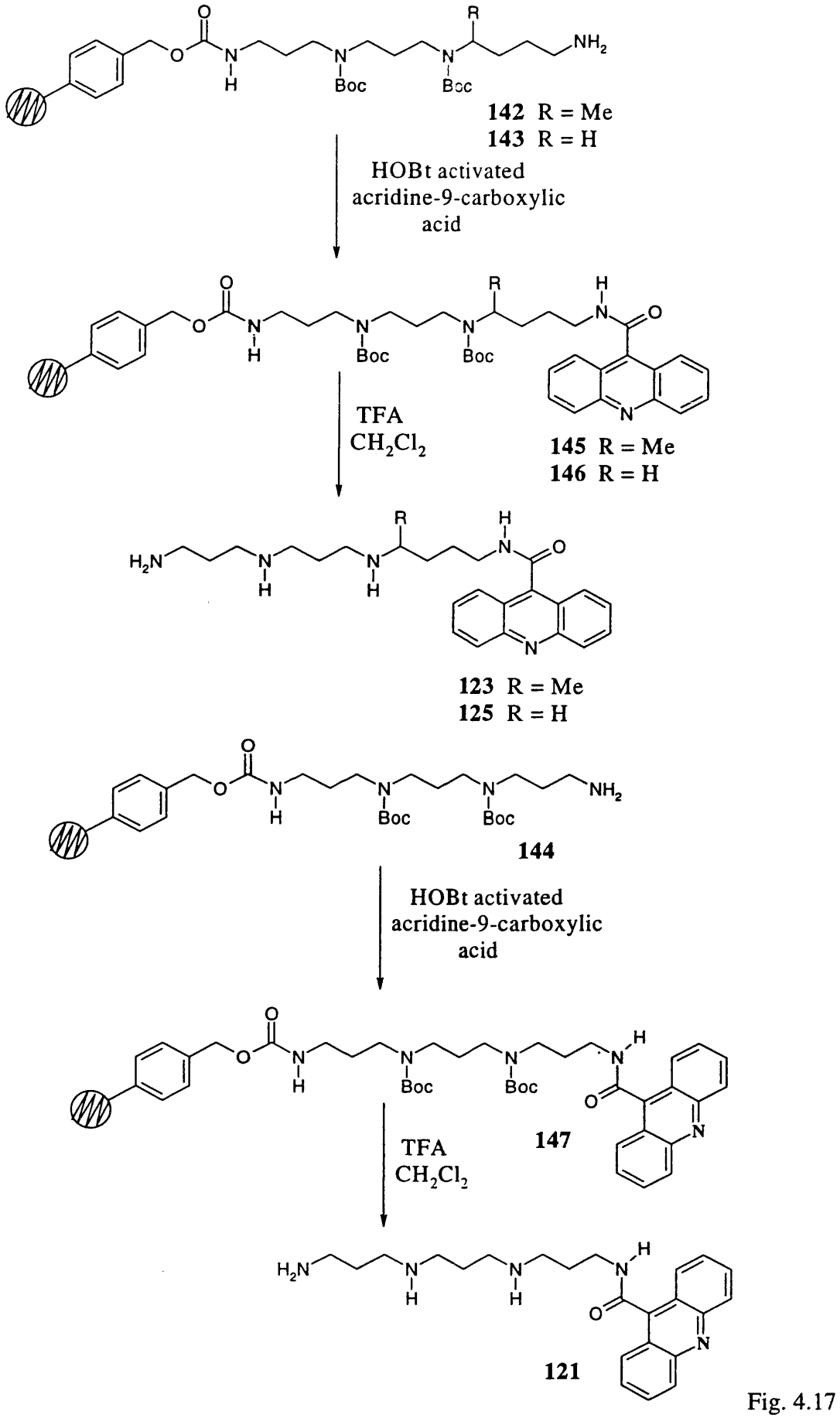

142 R = Me
143 R = H
HOBt activated
acridine-9-carboxylic
acid
TFA
CH_2Cl_2
145 R = Me
146 R = H
123 R = Me
125 R = H
144
HOBt activated
acridine-9-carboxylic
acid
147
TFA
CH_2Cl_2
121

Fig. 4.17

These three resins (**145-147**) were then exposed to TFA to bring about cleavage of the functionalised polyamines from the resin (Fig 4.17). The residue from each deprotection was purified by flash column chromatography on silica gel. Products were isolated from each resin with spectral characteristics fitting with the desired conjugates, **121**, **123** and **125**. Unfortunately the yields were low, a problem which was probably due to poor acylation reactions. The chromatography of the residues from the cleavages showed only one product in each case having both a polyamine and acridine moiety. If the problem had been due to slow or inefficient reductive amination reactions, then it would be expected that a quantity of the acylated triamine would also have been detected. Clearly, work still has to be done to investigate the problems associated with the acridine acylation step and to improve it to an acceptable level.

After the reductive amination steps, another synthetic route was followed towards the amides **122**, **124** and **126**. The resins **136**, **137** and **138** were acylated with HOBt activated acridine-9-carboxylic acid **130** in DMF as previously described then the protecting groups were removed (Fig. 4.18). In the case of the two azides **148**, **149**, this was by action of PPh_3 and in the case of the Pnt protected amine **150** this was by exposure to I_2. After washing and drying, these three resins **151-153** were subjected to cleavage by TFA. The residues from these reactions all showed uncoupled polyamines and none of the desired conjugates, **122**, **124** and **126**, by TLC and by ^{1}H-NMR. Again the problem is most likely due to failure of the acylation reactions. The exact reason why these reactions are so reluctant is not entirely clear although there may be problems of steric bulk preventing the conjugates forming in preferred conformations.

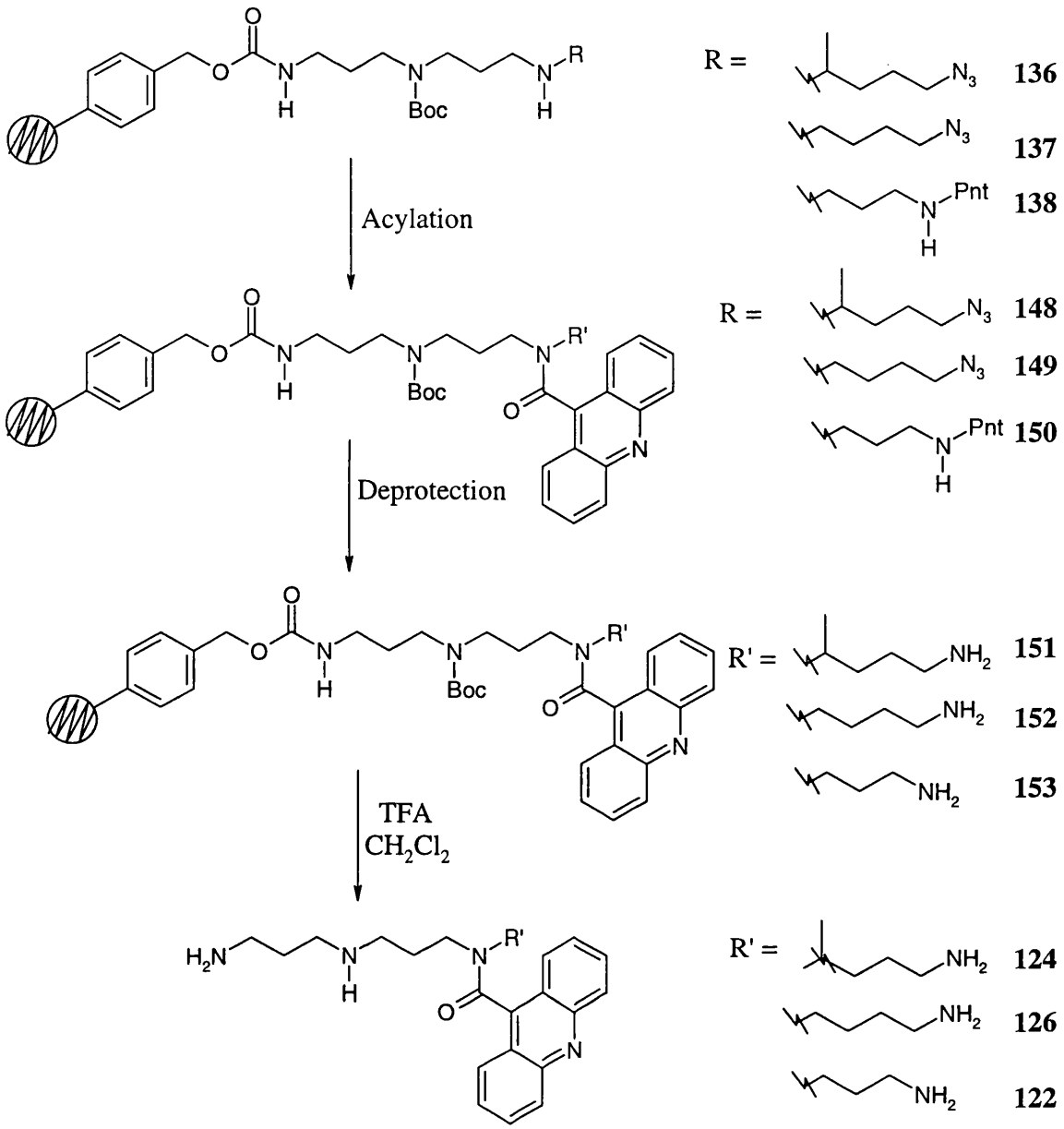


Fig. 4.18

Solid phase synthesis of polyamines is a quick and efficient way of making a larger number of compounds for biological evaluation. This study has proven the feasibility of building unsymmetrical polyamine chains whilst anchored to a solid support and the selective unmasking of amines to reveal selectively sites for conjugation. More time spent on improving the acylation stages reported in this work will complete the methodology for fast access to a variety of polyamine-acridine conjugates.

Chapter 5

Synthesis of 23-, 24- and 28-Membered Polyamine Containing Lactams

In Chapter 1 it was stated that a number of polyamine containing natural products and synthetic conjugates have potential in the treatment of certain parasitic infections. One specific target enzyme in trypanosomal parasites is trypanothione reductase (TR) which performs a crucial role in protecting the infective organism from oxidative stress produced by free-radicals (Walsh *et al.*, 1991; Fairlamb and Cerami, 1992). In Fig. 5.1 there is an outline of the biologically protective role TR plays in trypanosomal metabolism.

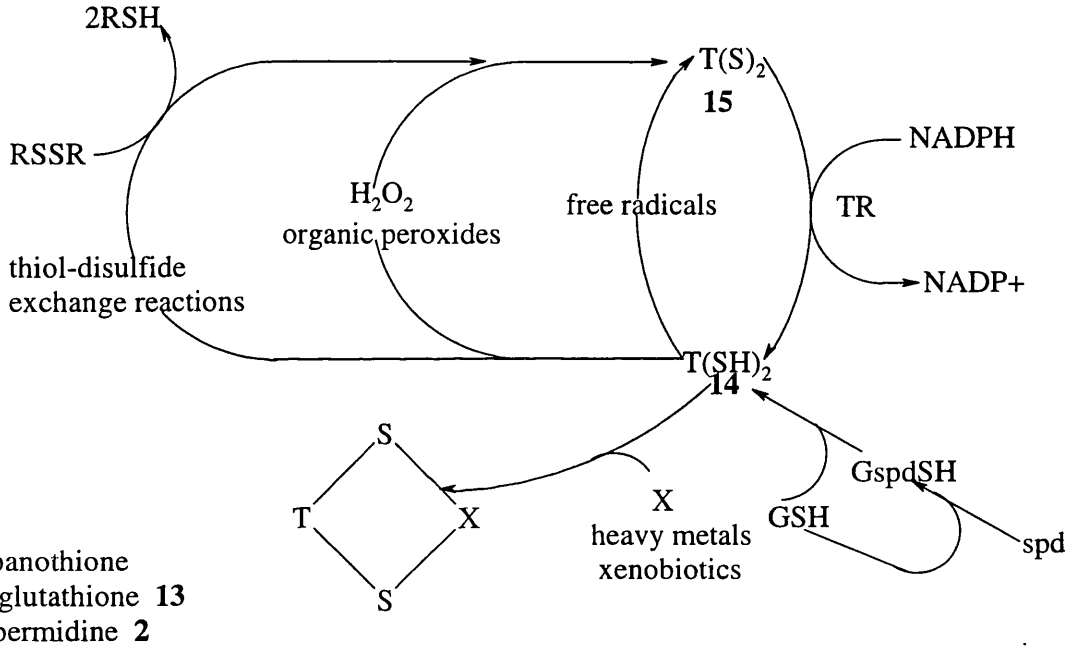
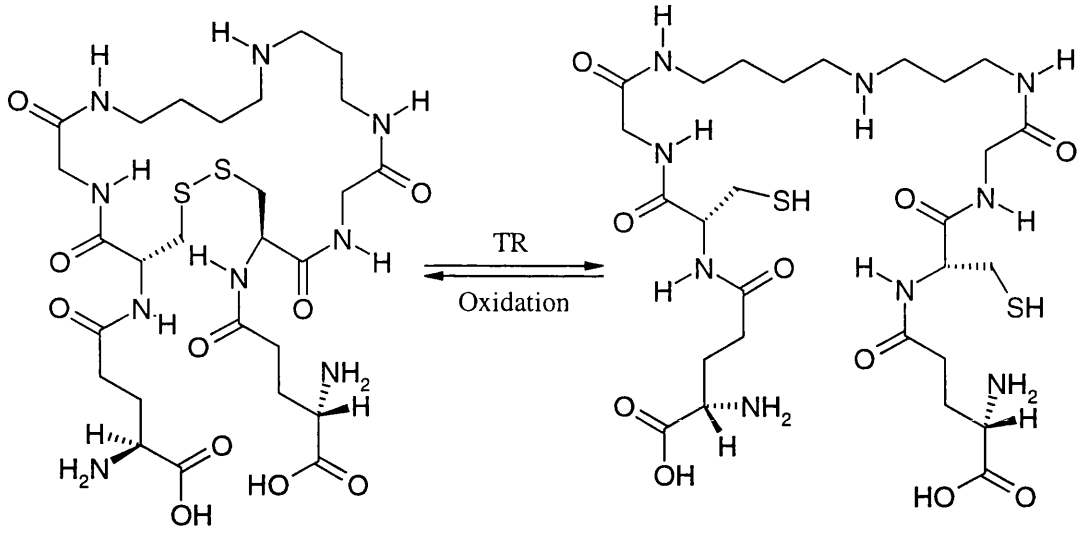


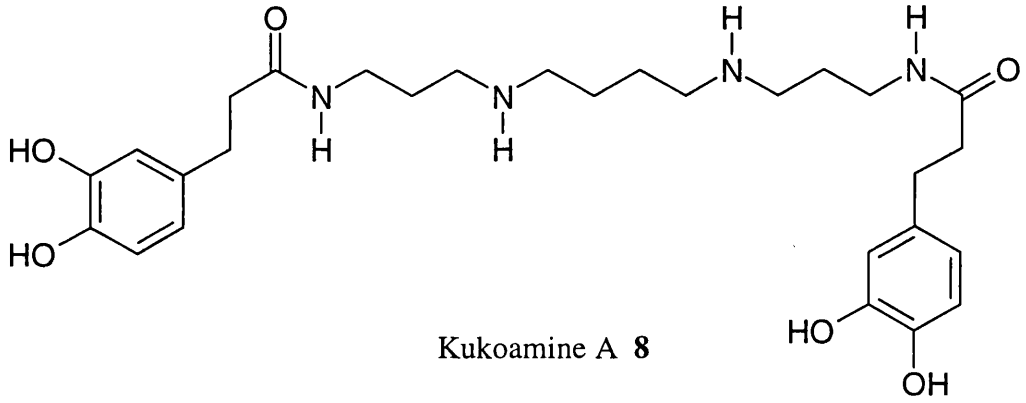
Fig. 5.1

(after Fairlamb and Cerami, 1992)

In the search for compounds which will inhibit TR, and hence have medicinal potential, the plant natural product kukoamine A has been identified as having useful activity (Ponasik *et al.*, 1995). A comparison between kukoamine A **8** and trypanothione disulfide **15**, the natural substrate for TR, reveals a number of key similarities and differences.

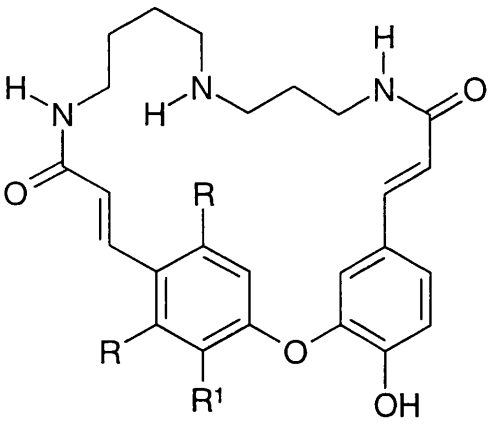


Trypanothione disulfide **15** Trypanothione **14**



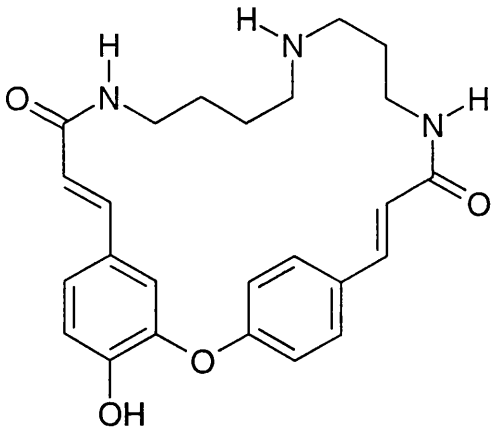
Kukoamine A **8**

Kukoamine A **8** is a positively charged spermine conjugate whereas trypanothione disulfide **15** is a positively charged spermidine conjugate. Both polyamine chains are terminally *N*-diacylated. Kukoamine A **8** has a largely open-chain structure whilst trypanothione **15** is a 24-membered macrocycle. Detailed searches of the literature reveal several compounds e.g. codonocarpine **154** (Doskotch *et al.*, 1971 and 1974), cadabicine **155** (Ahmad *et al.*, 1985 and 1987) and capparisine **156** (Ahmad *et al.*, 1987), all plant natural products, which contain spermidine moieties in 24-membered rings.



R = H, R^1 = OMe; Codonocarpine **154**

R = OMe, R^1 = H; Capparisine **156**



Cadabicine **155**

Similar to kukoamine A **8**, these compounds contain a polyamine which is acylated on the primary amines by cinnamic acid (3-phenylpropenoic acid) derivatives. In the case of the cyclic compounds **154-156**, a macrocycle has been formed by a diphenyl ether linkage bridging between two aromatic carbons, one from each cinnamate residue. It is interesting to note that whilst apparently structurally the same, apart from the pattern of oxygenation around the aromatic rings, there is in fact a difference in the carbon skeleton between cadabicine **155** and codonocarpine **154** and capparisine **156**. In cadabicine **155**, the propylamine residue in the spermidine **2** moiety has been acylated by a cinnamate which has a *para* substitution pattern between the vinylic carbon and the cyclic ether bearing carbon. This relationship is *meta* in the other two compounds **154** and **156**. If the acylation pattern is examined on the butylamine side of the spermidine moiety, then the pattern is reversed. These compounds have potential as inhibitors of TR. They will bear the same charge at physiological pH and they all are 24-membered rings (Fig. 5.2). Cadabicine **155** was chosen as the compound to be synthesised with the aim of testing it for TR inhibitory activity. It was first isolated from *Cadaba farinosa* in 1985 (Ahmad *et al.*, 1985) and has subsequently been isolated from *Crataeva nurvala* along with the (*N*- and *O*-) diacetate derivative (Ahmad *et al.*, 1987).

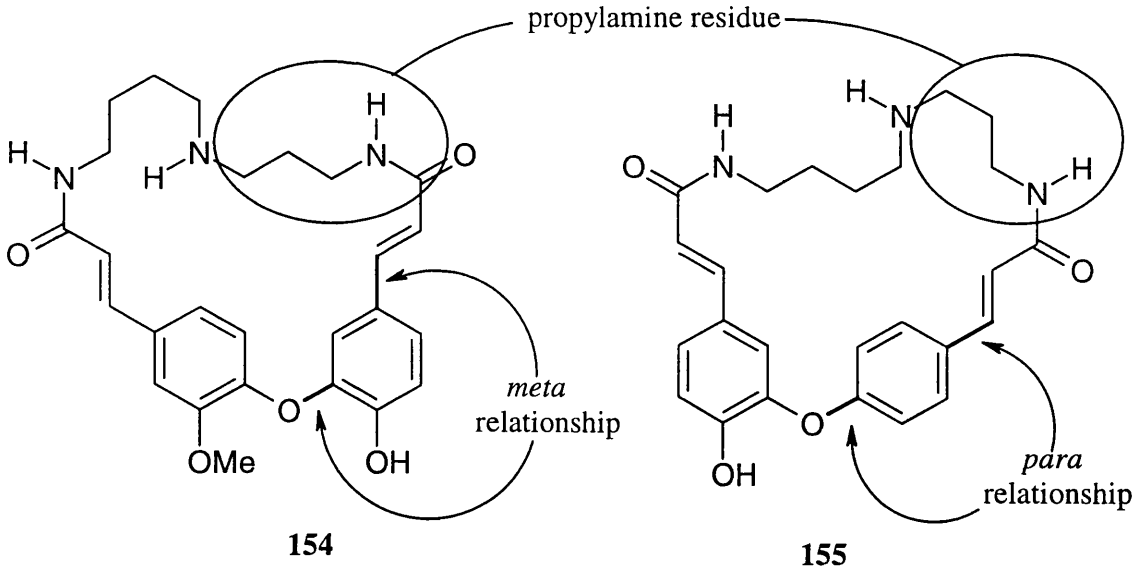
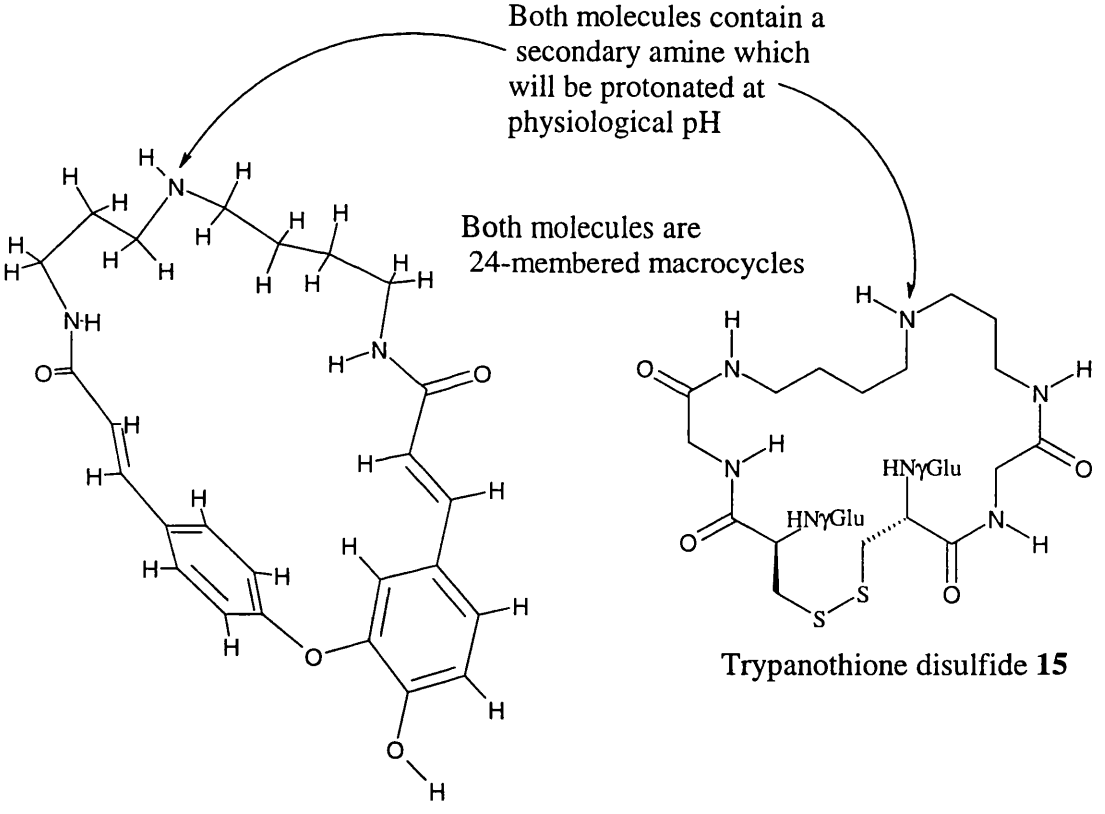


Fig. 5.2



Trypanothione disulfide **15**

Conformation of cadabicine **155**
from X-ray single crystal analysis
(Ahmad *et al.*, 1985)

Fig. 5.3

Both plants are commonly found in Pakistan, and parts of both have been reported to have medicinal properties. Previous studies have led to the isolation and identification of other alkaloids in these plants (Ahmad *et al.*, 1985). The structure of cadabicine **155** was identified by spectral methods including ^{1}H- and ^{13}C-NMR, IR, UV and mass spectrometry and finally an X-ray crystal structure (Fig. 5.3) was also obtained (Ahmad *et al.*, 1985).

There are a number of reasons why it is interesting and important to come up with a total synthesis of this natural product. From a chemical point of view, this compound possesses a number of synthetic challenges. The regiochemistry of the spermidine must be controlled as the target is unsymmetrical. There must be a method for differentiating between the two terminal amines. The diphenyl ether must be formed, a potentially difficult carbon-oxygen bond forming step. The macrocycle must be closed, an entropically unfavourable process. From a practical point of view, an efficient synthesis would allow the required amounts of compound to be easily to hand, as the naturally extracted product is scarce in the scientific community.

At the commencement of these studies, the literature was searched for procedures for forming diphenyl ethers. There are broadly three approaches which have been followed, thallium trinitrate promoted phenolic oxidative couplings (Rama Rao *et al.*, 1995), Ullmann reactions (Lindley, 1984; Rama Rao *et al.*, 1995) and S_NAr reactions involving the displacement of an *ortho*-nitro activated fluorine (Zhu, 1997).

Before the 1980's, the most commonly utilised method in the literature for the formation of diaryl ethers was by copper assisted couplings of phenols with aryl halides. The *ipso* displacement (i.e. attack at an aromatic position bearing a substituent) of aryl halides with nucleophiles was first reported in 1901 by Ullmann and has since been widely used and further modified. The variety of nucleophiles, reaction conditions and results from mechanistic studies have been reviewed by Lindley (1984). The simplest

examples of diaryl ether formation have been carried out with powdered copper as the catalyst. A typical example is the formation of 2-methoxydiphenyl ether. 2-Methoxyphenol is reacted with sodium hydroxide to give the corresponding phenolate which is dried and then heated to 200 °C in air for 2 h with bromobenzene and copper powder. The product is isolated in 62 % yield; this is greater if phenol is used and is often lower or negligible if reactants with greater functionality are used. The active catalyst in this reaction is thought to be a copper oxide which forms when the copper powder reacts with air, as copper(I) oxide and copper(II) oxide have both been shown to be better catalysts than elemental copper. Investigations have shown that a wide variety of copper compounds will catalyse this reaction and it has also been shown that the choice of base, solvent, temperature and duration of reactions can be crucial for getting the reaction to work, the exact combination depending upon the nature of the reactants. Mechanistic studies that have been carried out indicate the formation of a copper phenoxide. The aryl halide can then ligate with the copper bringing the reacting species into close proximity, the requirement for ligation possibly showing the need for careful solvent choice. The diagram below (Fig. 5.4) shows a mechanism proposed by Weingarten (1964).

The harsh reaction conditions often cause low yields due to the effects of substituents on the aromatic rings causing decomposition. Despite this, careful manipulation of reaction conditions has led to some utility in the synthesis of natural products including (±)-vertaline **157** (Hart and Kani, 1982) (Fig. 5.5). The diphenyl ether was accessed by preforming the desired copper phenoxide with NaH and CuI and treating it with either the bromide or iodide of the other aromatic species. The reaction was heated under reflux for 3 h in pyridine yielding the desired ether in 38 % yield along with recovered starting material and a small amount of an undesired reduction product.

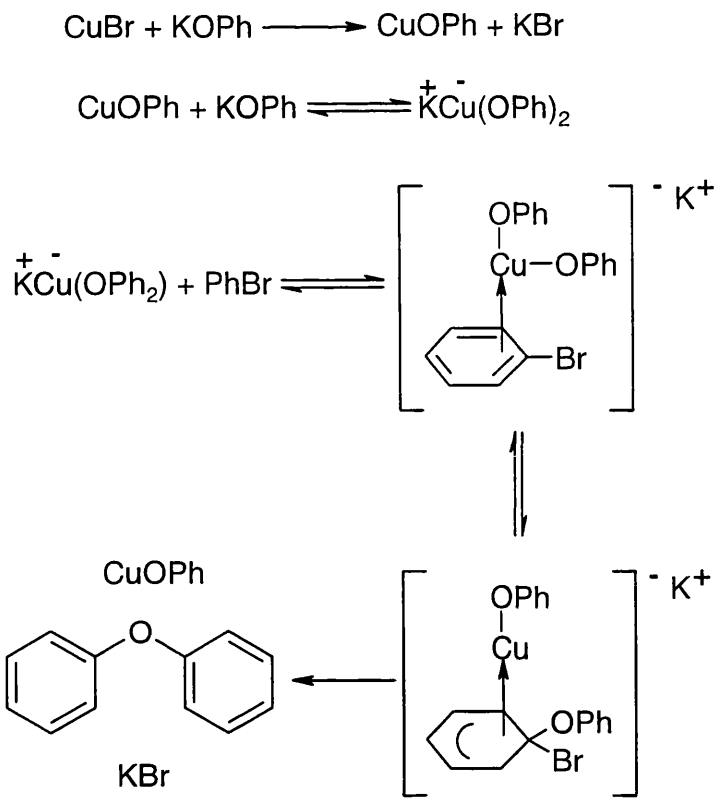
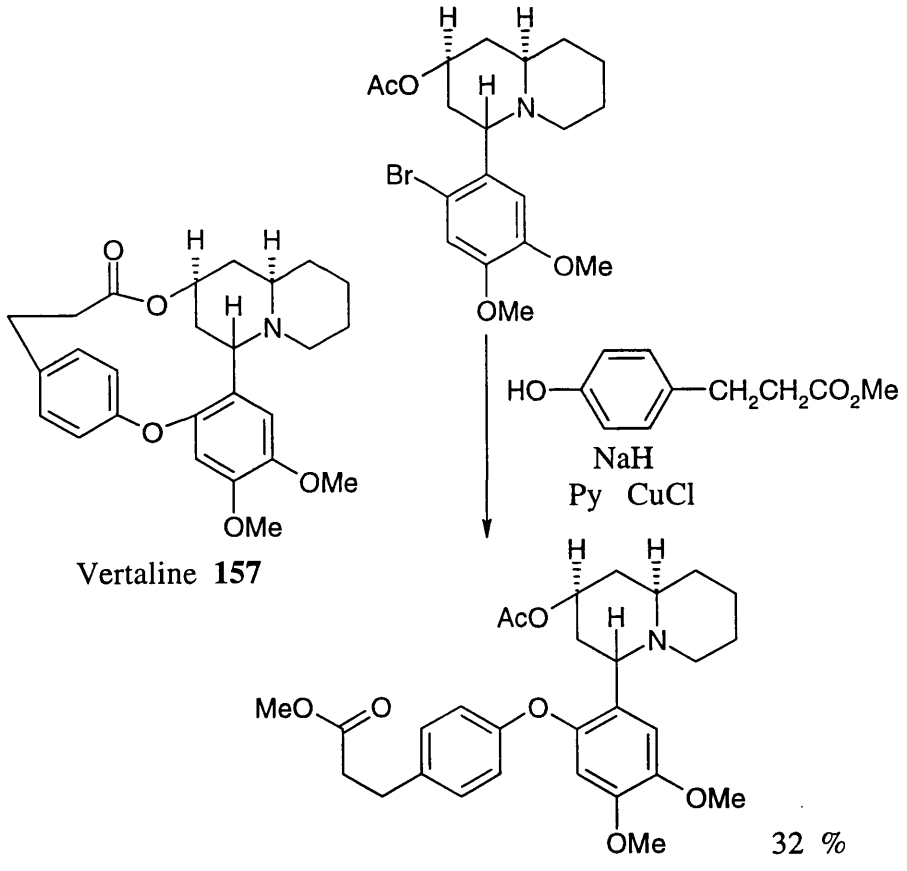


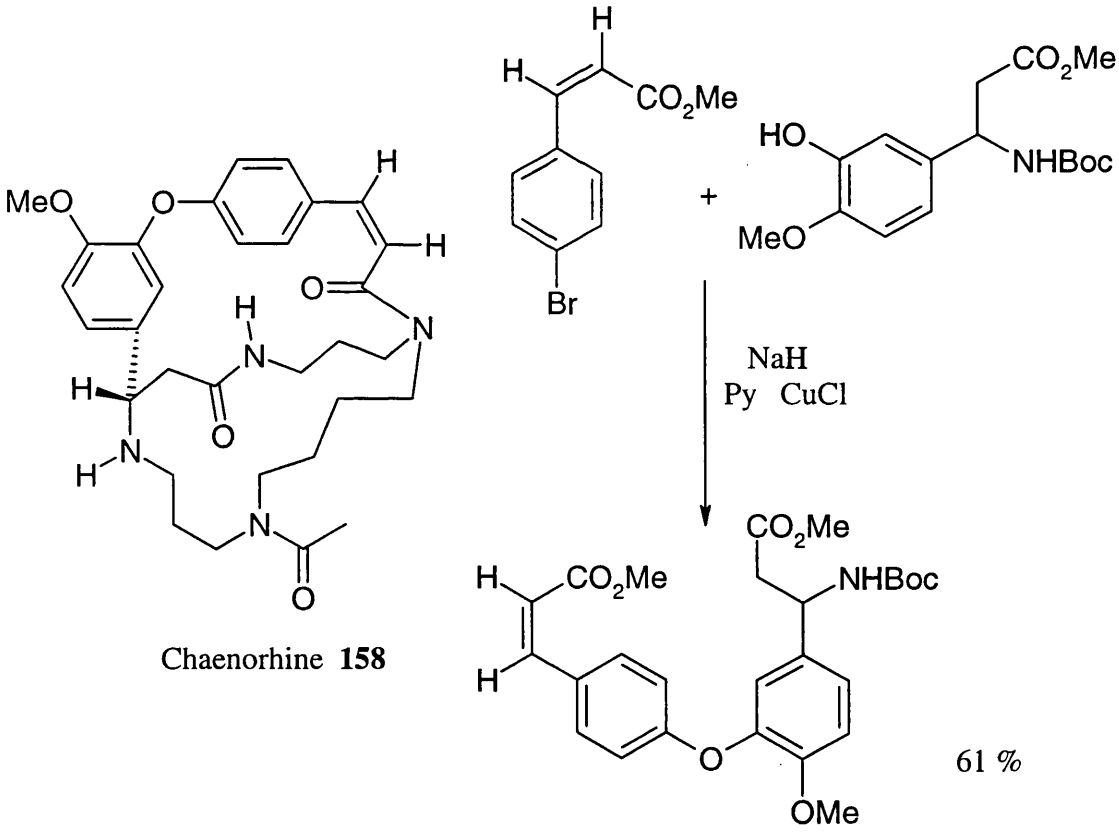
Fig. 5.4



Vertaline **157**

Fig. 5.5

Another elegant synthesis has been described by Wasserman and co-workers (1983); an Ullmann reaction is at the start of their synthesis of the natural product chaenorhine **158**. This compound is a polyamine conjugate with a certain structural similarity to cadabicine **155**. The diphenyl ether unit comes from two cinnamate moieties and the polyamine is spermine **1** which has been acylated on nitrogens N^1 and N^2 and has been acetylated on N^3. In the biosynthesis, the remaining primary amine appears to have come round and attacked one of the cinnamates in a 1,4-conjugate fashion to form two macrocycles and the associated oxygen has been maintained at the carbonyl oxidation level. In Wasserman's synthesis, Fig. 5.6, the diaryl ether was formed in 61 % yield by reaction between the required phenol and aromatic bromide in the presence of NaH, CuCl and pyridine heated under reflux. A preformed polyamine macrocycle containing an imidate ester was then conjugated to this diaromatic ether system.

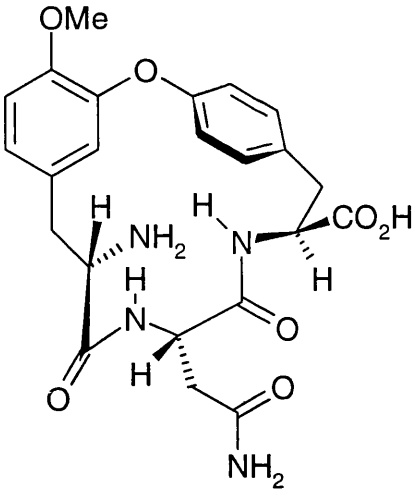


Chaenorhine **158**

Fig. 5.6

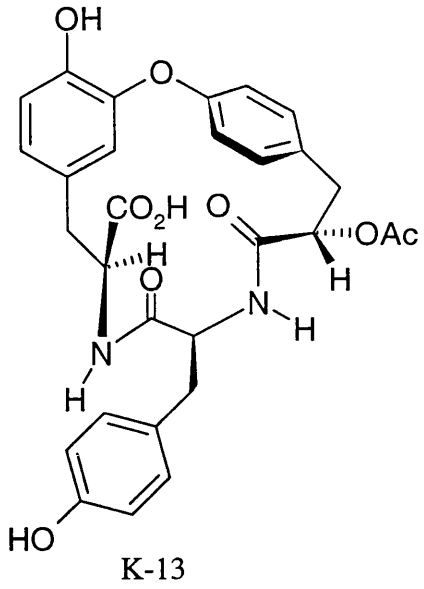
It is interesting to consider the biosynthesis of plant natural products such as chaenorhine. Many compounds have been isolated from plant sources containing spermine and spermidine and amongst these a large subset are acylated on one or more amine groups by cinnamic acids (Guggisberg and Hesse, 1983 and 1998). These cinnamamides have a variety of patterns of oxidation around the six-membered aromatic rings. Further biosynthetic reactions add to structural diversity. Kukoamine A **8** is a spermine **1** conjugate which has been acylated twice by caffeic acid (3,4-dihydroxy cinnamic acid) and then the alkene bonds have been reduced. Chaenorhine **158** is a spermine conjugate which has been acylated on amines 1 and 2 by two cinnamic acids which have then undergone an etherification to form a diaryl ether link. Amine 3 has been acetylated and amine 4 has performed a Michael-type (1,4) addition to an alkene in one of the cinnamates to form a further ring.

Recent synthetic work has widened the utility of the Ullmann reaction as a number of researchers have addressed the synthetic challenges provided by a number of natural peptidic compounds containing oxidatively coupled residues, normally derived from two tyrosine units (Rama Rao *et al.*, 1995). Not only are these compounds of interest to the organic chemist, but they also often display good antibiotic and anticancer biological activities. For a review of a number of synthetic methods towards these compounds, see: Rama Rao *et al.* (1995). Evans and co-workers have published several syntheses of peptidic compounds containing diaryl ethers, e.g. in 1989 the syntheses of tripeptides OF4949-III **159** which is of interest as an anti-cancer agent and K-13 **160** which is an ACE inhibitor (Evans and Ellman, 1989). These 17-membered macrocycles were built from a common diphenyl ether unit **161** produced from the intermolecular Ullmann reaction between two differentially protected cinnamate esters. The remainder of the rings were built up by established peptide methodologies.



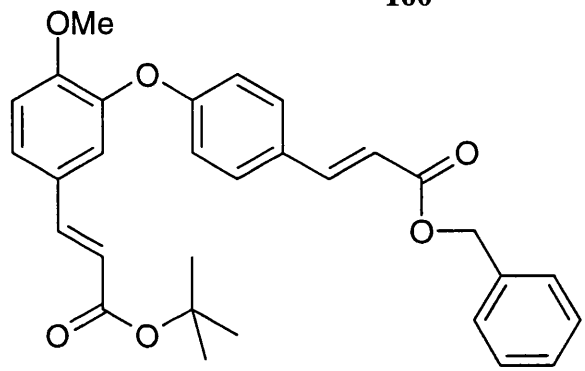
OF4949-III

159



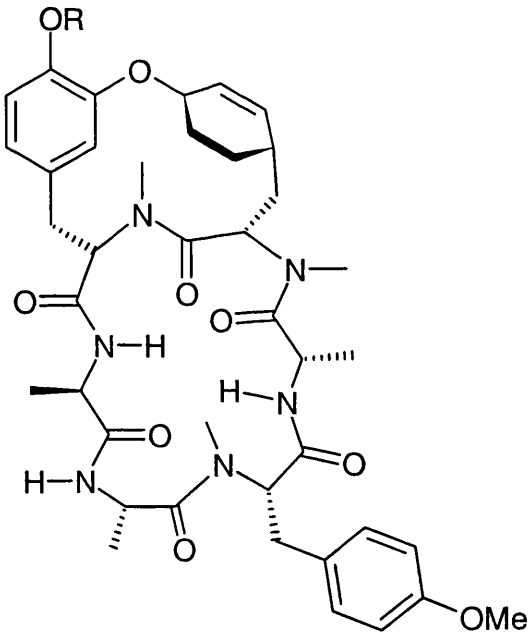
K-13

160



Common building block

161



RA-VII (R = Me) **162** and Deoxybouvardin (R = H) **163**

Further work has been published by Boger and co-workers relating to the synthesis of the antitumour antibiotics RA-VII **162**, deoxybouvardin **163** and related compounds (Boger *et al.*, 1993; Boger and Zhou, 1995). These hexapeptides contain a 14-membered cyclophane and an 18-membered peptide ring (Boger *et al.*, 1992). The initial approach taken was to form a diaryl ether linkage as an open-chain, and then to use a peptide coupling to close the small, 14-membered ring before attaching the larger peptide ring (18-membered) (Boger *et al.*, 1993). This approach failed as the small ring **164** could not be closed, the only product being dimeric, formed from two of the diaryl units **165** (Fig. 5.7). Success was achieved by using the Ullmann reaction as a macrocyclisation step to form the 14-membered ring **166**. In all cases the base used was NaH, but varying the substituents on the reactants led to more efficient reactions with different catalyst and solvent combinations (Boger *et al.*, 1993).

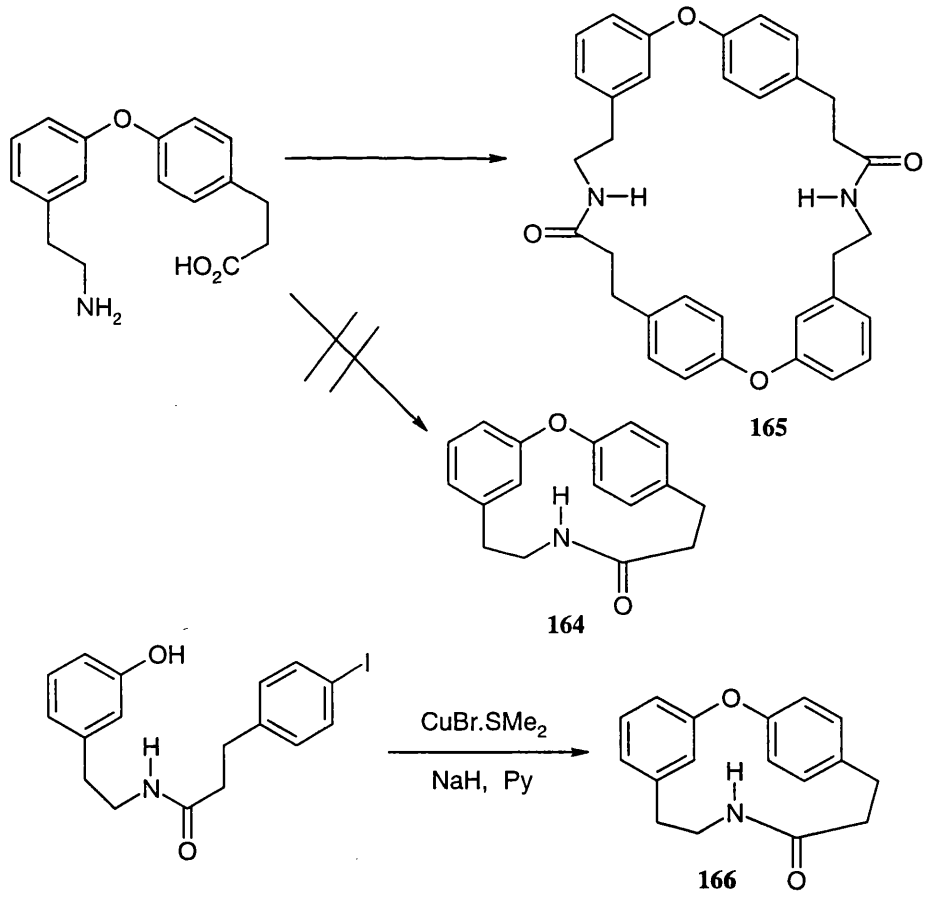
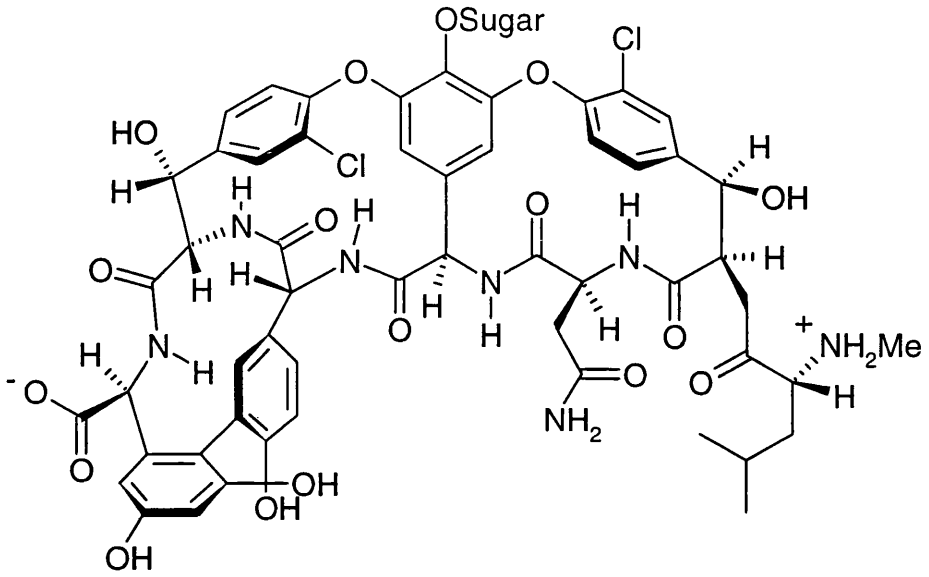


Fig. 5.7

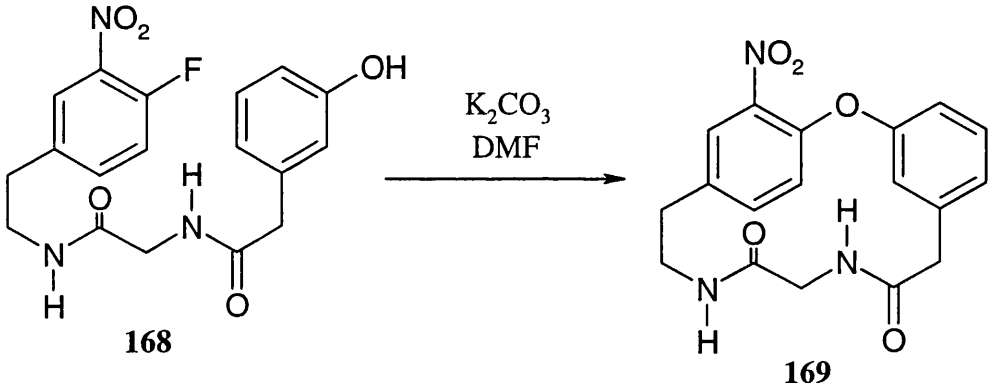
From the time this work was published, the majority of syntheses of diphenyl ether containing natural products have used the ether bond formation as the cyclisation step. Boger has described the synthesis of related ring systems including models of rings in the antibiotic vancomycin **167** and related compounds (Boger *et al.*, 1993).



Vancomycin **167**

Vancomycin is an arylglycine rich heptapeptide which is conjugated to sugar residues. It is one member in a family of such antibiotics which differ in the peptidic structure as well as in the nature of the sugar groups appended. Vancomycin is currently used as a last resort against bacterial infections resistant to other antibiotics, although recently certain bacterial strains are appearing with vancomycin resistance. The elaborate structure has long been of interest to chemists as a synthetic challenge. The interaction between vancomycin and proteins has been well investigated by Williams and co-workers, largely through NMR studies (Williams, 1984, Williamson *et al.*, 1984). With the recent advance in diaryl ether forming methodologies, successive fragments of the structure have been prepared and this work has culminated with the publication, as this thesis nears completion, of the total syntheses of vancomycin aglycone simultaneously by the research groups of Evans (Evans *et al.*, 1998a and b)

and Nicolaou (Nicolaou 1998a, b and c). The key to making the diphenyl ether rings in vancomycin **167** has not been by the use of Ullmann reactions, but by S_NAr substitution reactions. Zhu and co-workers have pioneered the development of macrocyclisations using the displacement of an *ortho*-nitro activated fluorine with a phenolic oxygen (**168** to **169**). Zhu has published applications of this methodology towards the synthesis of ring fragments of vancomycin **167** (Beugelmans *et al.*, 1996; Vergne *et al.*, 1997) and the structurally related antibiotic teicoplanin (Bois-Choussy *et al.*, 1996 and 1997) as well in the synthesis of K-13 **160** (Beugelmans *et al.*, 1994) and deoxybouvardin **163** (Bigot *et al.*, 1997).



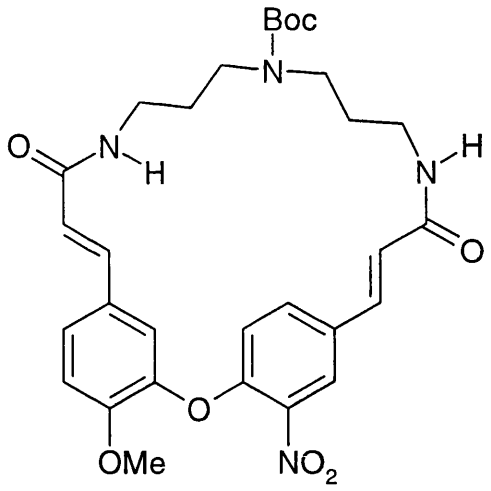
168 **169**

The advantages of this reaction over cyclisations by the Ullmann method are that the reaction is mild, requiring no more than a weak base in a polar, aprotic solvent to bring about reaction, and it is high yielding once the correct combination of base and solvent are established. There has been speculation about the mechanism of this reaction and why it proceeds so favourably along an entropically disfavourable path. It was initially proposed that there might be a π-π stacking interaction between the electron rich phenolic ring and the electron deficient fluoro-nitro ring. Computer modelling studies have been published predicting such an interaction showing the reacting centres to be close enough together to promote reaction (Zhu, 1997). However, there are two pieces of evidence which dispute this as being the key interaction driving the reaction. Firstly, these cyclisations can be run at concentrations (up to 1 M)

significantly higher than typical macrocyclisation conditions, e.g. typically reaction concentrations of 0.004 M have been reported by Boger for Ullmann ring formations (Boger *et al.*, 1993). Despite using a high concentration reaction mixture, the sole product is the intramolecularly formed ring and no intermolecluar dimerisation or polymerisation as might be expected. The second piece of recent evidence is the fact that aryl-alkyl ether bonds can be formed between alcohols and aromatic *ortho*-nitro fluorines. These reactions have been demonstrated to proceed in good yield, without the benefit of any interactions between the two aromatic moieties (Laïb and Zhu, 1998).

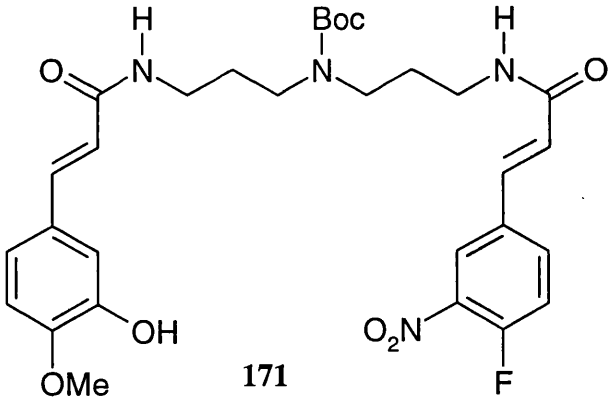
A literature search was performed for syntheses of cadabicine **155** and no successful procedures were found. Some work has been published on the synthesis of codonocarpine **154** using an Ullmann reaction to build the diphenyl ether unit followed by coupling of regioselectively protected N^1, N^2-di-Boc-spermidine (Humora *et al.*, 1980). Cleavage of the Boc groups and an amide forming step yielded the required 24-membered macrocycle. Other workers had followed a similar route towards cadabicine, but failed to bring about the lactamisation step (Nogradi *et al.*, 1994). As the majority of these published diphenyl ether forming macrocyclisations (*vide supra*) are for ring sizes in the 14-18 membered range, it was of interest to see if such methodology could be applied to forming larger rings sizes. In making small rings (3- or 4-membered), the energetics of forming strained systems is disfavouring whereas in a larger system it is the entropic difavourability of bringing the widely spaced (remote) ends together which could be the limiting factor.

Our initial model studies using the Ullmann approach to the cyclisation were disappointing, the reactions being low yielding with the formation of a lot of brown decomposition products. These made chromatography difficult and any interesting products formed were not easy to purify. Due to these problems it was decided to look at the S_NAr approach to the synthesis.



170

In the first instance, rather than trying to make a spermidine containing ring, the symmetrical triamine *N*-(3-aminopropyl)-1,3-propanediamine **178** was substituted making the target a 23-membered macrocycle **170**. Using this as a model compound simplified the synthesis, as the issue of the regiochemistry of the triamine was not relevant at this stage. This compound has a methoxy group in place of the hydroxy in cadabicine **155**, a necessary protecting group which would be removed in the final synthesis of the natural product, and a nitro group which is required to activate the adjacent carbon for nucleophilic substitution. A Boc group is protecting the secondary amine, a precaution to prevent any side-reactions in the cyclisation step.



171

The open-chain precursor **171** to the target **170** requires the terminal diacylation of the suitably protected triamine by two cinnamic acids. 3-Hydroxy-4-methoxycinnamic

acid (isoferulic acid) **173** was synthesised by Knoevenagel condensation following literature procedures (Blase and Banerjee, 1995).

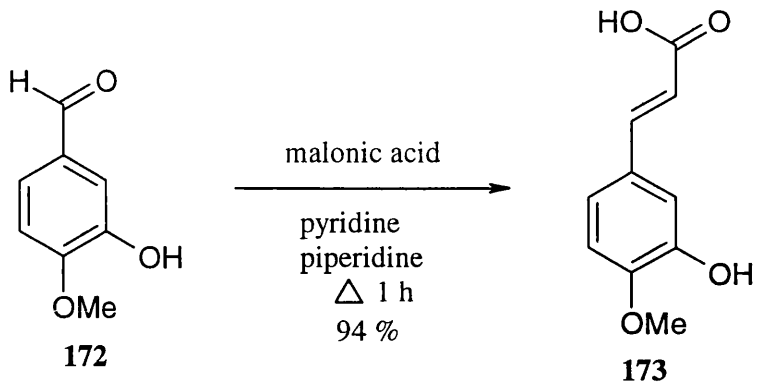


Fig. 5.8

3-Hydroxy-4-methoxybenzaldehyde **172** was heated to reflux for 1 h with malonic acid and piperidine in pyridine to give the desired *trans* cinnamic acid **173** in 92 % yield (Fig. 5.8). The ^{1}H-NMR spectrum proved the isolated cinnamic acid to be the desired *trans* rather than the *cis* isomer as signals at δ 6.24 and 7.45 ppm, corresponding to the two alkene protons, are split by 16 Hz. Typically *trans* coupled protons give coupling constants between 12 and 18 Hz whilst those with a *cis* relationship display 0-12 Hz (Williams and Fleming, 1987). In order to make the 4-fluoro-3-nitrocinnamic acid **176**, first 4-fluorobenzaldehyde **174** was nitrated under standard conditions giving only the desired mono-nitrated product **175**. Unfortunately, attempted Knoevenagel condensation with this aldehyde gave many products as visualised by TLC, so it was decided to change the order of the reactions. 4-Fluorobenzaldehyde **174** was smoothly converted into the cinnamic acid **177** by the standard procedure and was then nitrated by the action of conc. nitric acid giving the required *ortho*-fluoronitrophenyl substitution pattern **176** in 85 % yield.

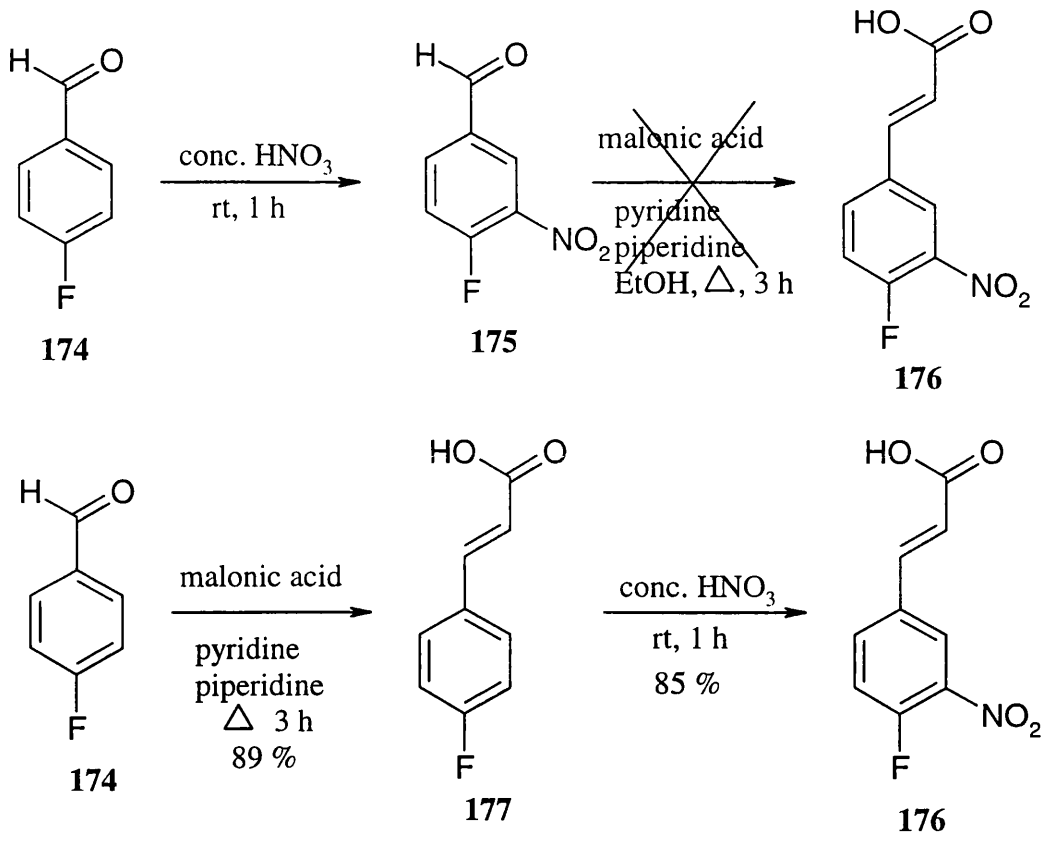


Fig. 5.9

For the protection of *N*-(3-aminopropyl)-1,3-propanediamine **178**, the trifluoroacetyl protecting group was used to discriminate between the primary and secondary amines (Fig. 5.10). Trifluoroacetyl, when introduced by stirring the triamine with ethyl trifluoroacetate, has overall selectivity for primary amines (O'Sullivan *et al.*, 1995; Xu *et al.*, 1995). Two equivalents were stirred with the triamine **178** in THF for 1 h at 25 °C before Boc anhydride was added (Fig. 5.10). This was allowed to react with the unmasked secondary amine **179** for 18 h, after which time conc. aq. NH_3 was added to the reaction mixture until the pH was above 11. The action of the NH_3 cleaves the trifluoroacetyl groups, but not the Boc group which is base stable and acid labile. After 24 h, TLC indicated that the reaction was complete, so it was worked up and the N^2-mono-Boc-triamine then isolated by column chromatography in 62 % yield **181**.

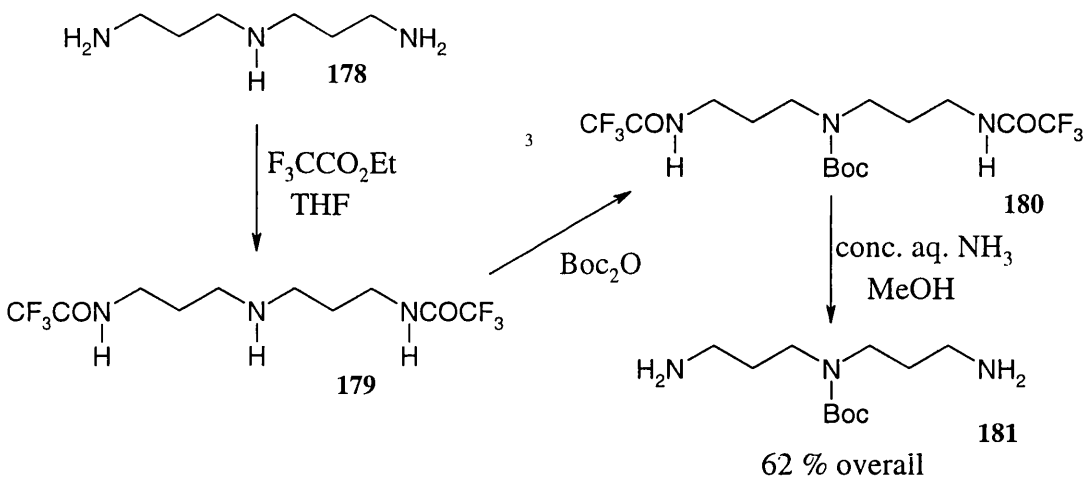


Fig. 5.10

Cinnamic acids **173** and **176** were then coupled to the triamine in a sequential fashion. First attempts were with isoferulic acid **173** using DCC and catalytic HOBt using 2 equiv. of the diamine **181** and dilute reaction mixtures to favour the mono-acylated product **182**. This reaction was slow and low yielding, so other possibilities for making the amide bond were investigated. Attempts to activate the acid by conversion into its acid chloride by action of thionyl chloride and catalytic DMF were not successful as the reaction created a number of by-products when investigated by TLC. Coupling was also tried by activating the acid as its pentafluorophenyl ester (DCC, HOBt, pentafluorophenol). Couplings with this reagent gave approximately the same yield as DCC alone. The best method tried was by using 2-mercaptothiazoline as an activating agent (Nagao *et al.*, 1980 and 1984) (Fig. 5.11). The amides formed from acids by treatment with this reagent (in this case by action of DCC/catalytic DMAP) are susceptible to nucleophilic attack by primary amines. These compounds are also bright yellow, this colour disappearing when these intermediates are quenched with amines allowing the progress of a coupling reaction to be visually assessed. In this instance, the 2-mercaptothiazoline activated isoferulic acid was synthesised in 1 h with 1.5 equiv. of DCC, a catalytic amount of DMAP and 1.1 equiv. of 2-mercaptothiazoline. Once the yellow colour had appeared, and TLC indicated all of the starting materials had been

consumed, the reaction mixture was filtered to remove the insoluble DCU, and the filtrate was added immediately to a stirred solution of 2 equiv. of the diamine **181**. After 10 h the yellow colour had disappeared and TLC indicated that the reaction had run its course. After careful flash column chromatography on silica gel, the desired amide **182** was isolated in 75 % yield.

4-Fluoro-3-nitrocinnamic acid **176** was coupled to the remaining primary amine using the same protocol except this time a 1:1 ratio of activated acid to amine was used. The cyclisation precursor **183** was recovered in 69 % yield after chromatography.

Macrocyclisation was bought about by use of a standard procedure developed towards the synthesis of vancomycin fragments (Zhu *et al.*, 1997) (Fig. 5.11). The precursor **183** was stirred in anhydrous DMF with 5 equiv. of CsF for 18 h. TLC showed the formation of a slightly more polar product which stained a different colour when exposed to $FeCl_3$. This compound was isolated by chromatography on silica gel and was examined spectroscopically. The ^{1}H-NMR shows a change in the splitting in the aromatic region. Signals which displayed fluorine coupling in the open chain analogue no longer did after the cyclisation. The proton on the carbon *ortho* to the carbon bearing fluorine was a doublet of doublets at δ 7.71 ppm showing a 2 Hz coupling to fluorine and a 8 Hz coupling to an *ortho* proton. After cyclisation this signal displays only the proton-proton coupling. The ^{13}C-NMR spectra also give an indication of the change which has occurred. In the precursor, the aromatic carbon attached to fluorine has an obvious signal at δ 158.2 ppm which is split into a doublet of 258 Hz. This signal has moved and there is no sign of this, or any of the carbons which previously displayed C-F coupling patterns, being split. The fluorine has been displaced by the phenolic oxygen. To confirm this the FAB-MS and FAB-HRMS of desired product **170** are both consistent with the cyclised product.

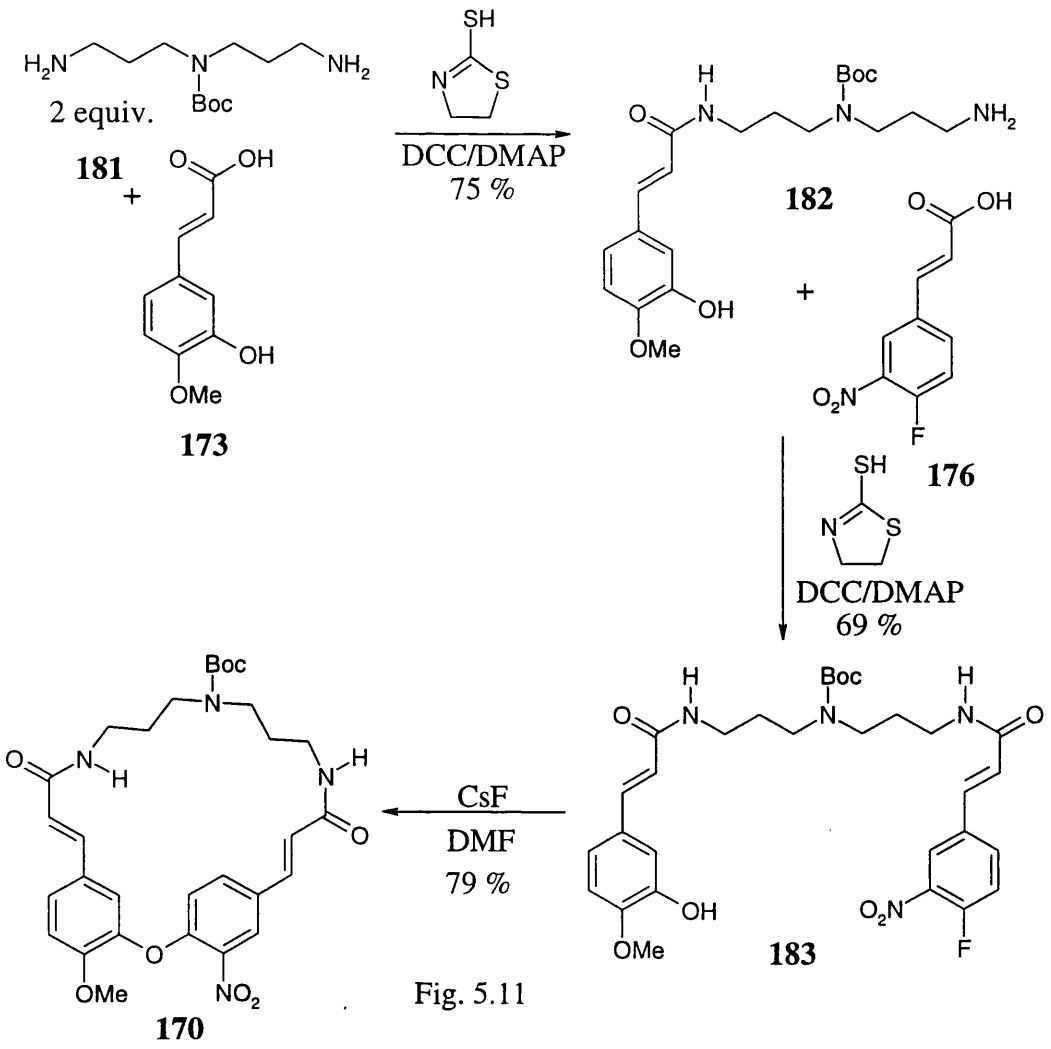


Fig. 5.11

After the success of this 23-membered ring formation, attention was turned to the 24-membered ring as found in cadabicine. To protect the spermidine required in this synthesis, the published formation of a hexahydropyrimidine adduct with formaldehyde was exploited (Ganem, 1982). The favourable formation of this 6-membered ring **184** occurs when spermidine **2** is stirred for 1 h with 0.95 equiv. of aq. formaldehyde in a quantitative fashion Fig. 5.12. This adduct allows for the differentiation of the primary amines in spermidine as in this cyclic form there are two amines which can be acylated, a primary and a secondary, and these have different nucleophilicities. The more nucleophilic primary amine was reacted with the 2-mercaptothiazoline activated isoferulic acid at an initial temperature of -78 °C. On addition of the acylating agent, the cooling bath was removed and the reaction was allowed to warm to 20 °C.

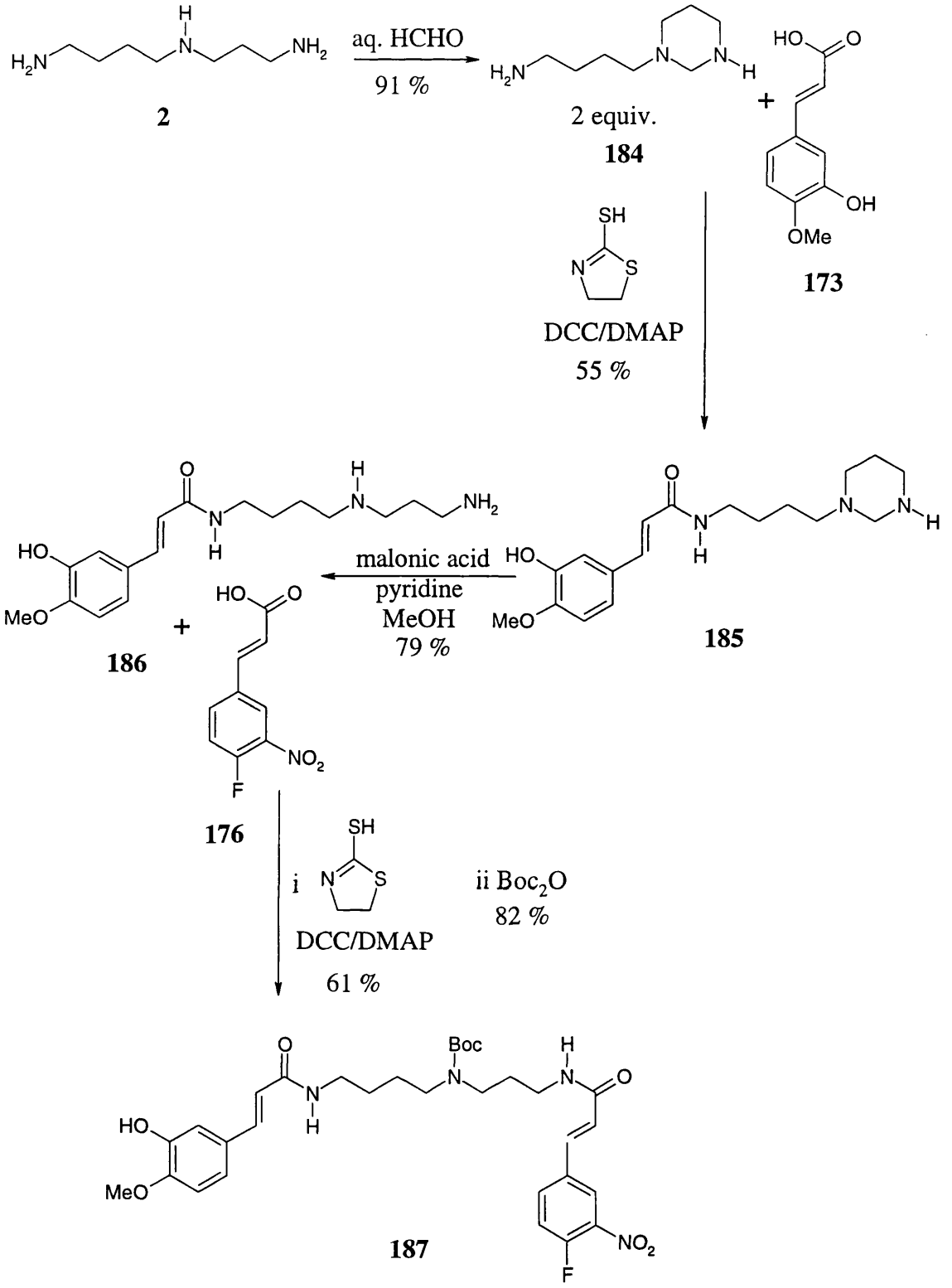


Fig. 5.12

The cold temperature was employed to ensure the kinetically favoured primary acylation occurred rather than acylation at the secondary amine. The desired amide **185** was recovered after chromatography in 55 % yield. The hexahydropyrimidine ring was then opened by application of the Knoevenagel procedure, heating the adduct with malonic

acid and pyridine in anhydrous EtOH for 18 h. The monoacylated spermidine **186** was recovered in 79 % and was then acylated with activated 4-fluoro-3-nitrocinnamic acid **176** in 61 % yield (Fig. 5.12).

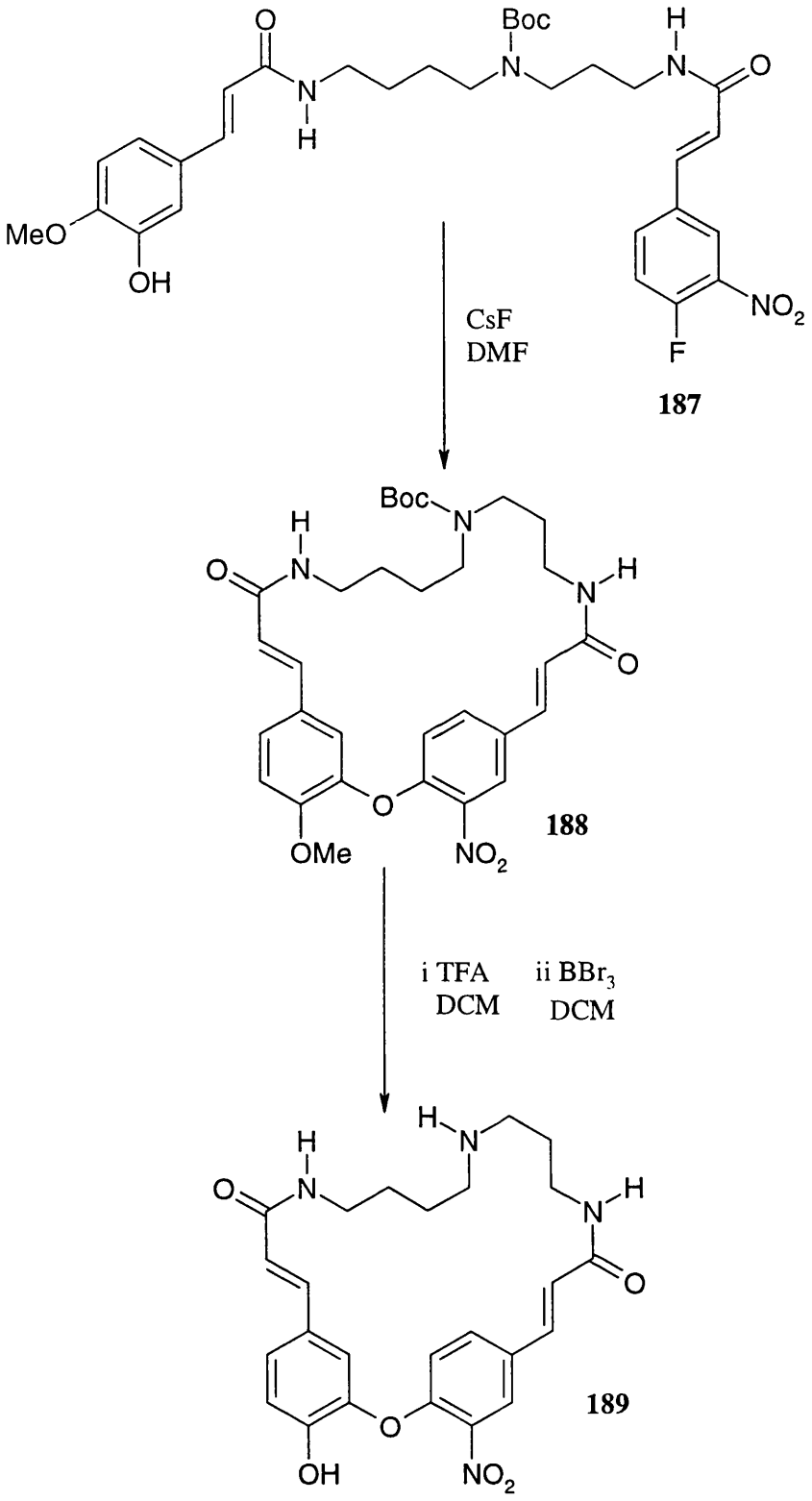
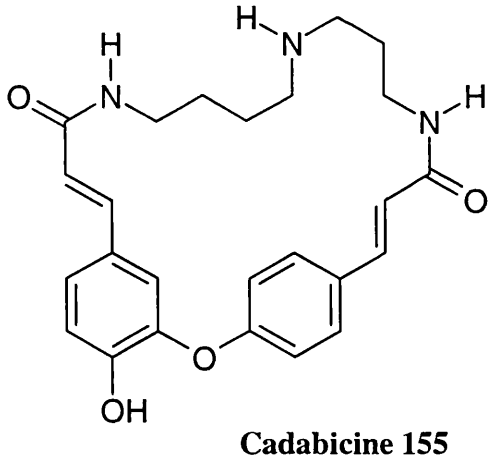
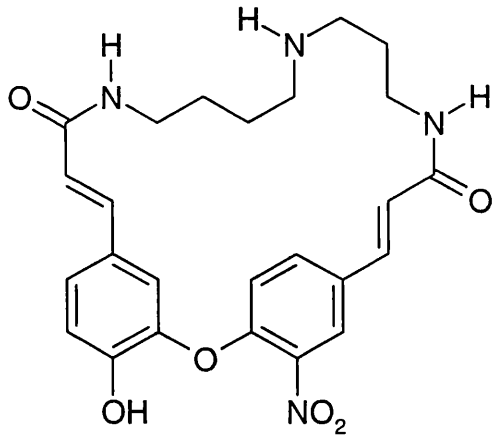


Fig. 5.13

Before cyclisation the secondary amine was protected with a Boc group by reaction with Boc anhydride (89 %) to ensure that the free amine did not interfere with cyclisation. As with the 23-membered ring **170**, CsF mediated cyclisation this time in 71 % yield in DMF (Fig. 5.13). The reagents had to be thoroughly dried before reaction or the starting material **187** remained unreacted. That the cyclisation had occurred was confirmed by TLC and NMR spectroscopy as well as by reverse-phase HPLC in which the residual reactant **187** was clearly seen separately to the product **188** peak. There was a significant line broadening in the ^{1}H-NMR signals in the cyclised compared to the non-cyclised compound which could be expected due to the loss of free rotation in the macrocycle. Mass spectroscopy and HRMS were also used to confirm the identity of the product (595.2793 found, 595.2768 required for MH^{+}),. TFA was used to cleave the Boc group from the secondary amine and then attention was turned to the methyl ether. In cadabicine there is a hydroxyl at this position so an *O*-demethylation step is required. Initially reactions were attempted with TMSI, but despite trying a range of temperatures, the reactions gave either recovered starting material or decomposition products. The transformation was successfully mediated by action of BBr_3 over 3 h at -78 °C. After chromatography, the nitro-cadabicine analogue **189** was isolated in 60 % yield.



Cadabicine 155



189

This compound **189** is a key intermediate in the total synthesis of cadabicine **155**, requiring only the removal of the nitro group. It provides the opportunity for structure-

activity studies to be performed with cadabicine analogues, particularly screening for inhibitors of TR. The final transformation into cadabicine could be achieved by careful reduction of the nitro to an aniline functionality (without reducing the olefins) and then a deamination step.

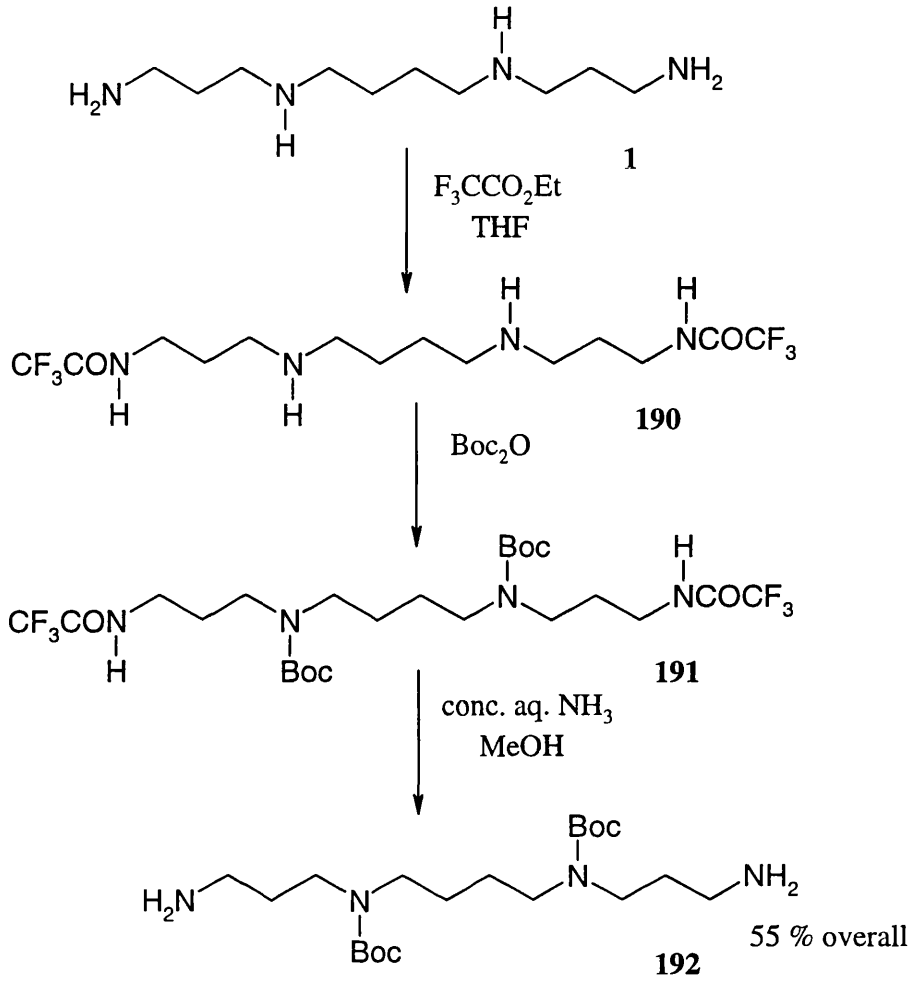


Fig. 5.14

Having synthesised 23- and 24-membered polyamine amide macrocycles there remained the question "how big can these rings be?" As a step towards answering this question, a target compound **194** was designed having the tetra-amine spermine **1** in place of spermidine **2** in the ring, thus making it a 28-membered macrocycle. The synthesis of the cyclisation precursor was analogous to the strategy employed with *N*-(3-aminopropyl)-1,3-propanediamine **178** since spermine is symmetrical (Fig. 5.14).

N^2,N^3-DiBoc-spermine **192** was isolated in 55 % yield and was then sequentially coupled to the two acids, 3-hydroxy-4-methoxycinnamic acid **173** then 4-fluoro-3-nitrocinnamic acid **176**. Attempts to bring about cyclisation were at first unsuccessful. No reaction was observed with CsF both in DMF and DMSO so the base was changed to K_2CO_3. No reaction was observed in DMSO until it was run with the addition of 18-crown-6 when there was a slow formation of a product spot as shown by TLC. It is assumed that this reagent is enhancing the nucleophilicity of the oxygen which was previously too low for reaction. As there was still a significant quantity of starting material **193** left after 24 h the reaction was re-run at 50 °C at which temperature cyclisation was complete after 5 hours (Fig 5.15). The macrocycle **194** was identified spectroscopically (NMR, FAB-MS, FAB-HRMS, found 752.3895, 752.3871 required for (MH^+)) and chromatographically (TLC, HPLC).

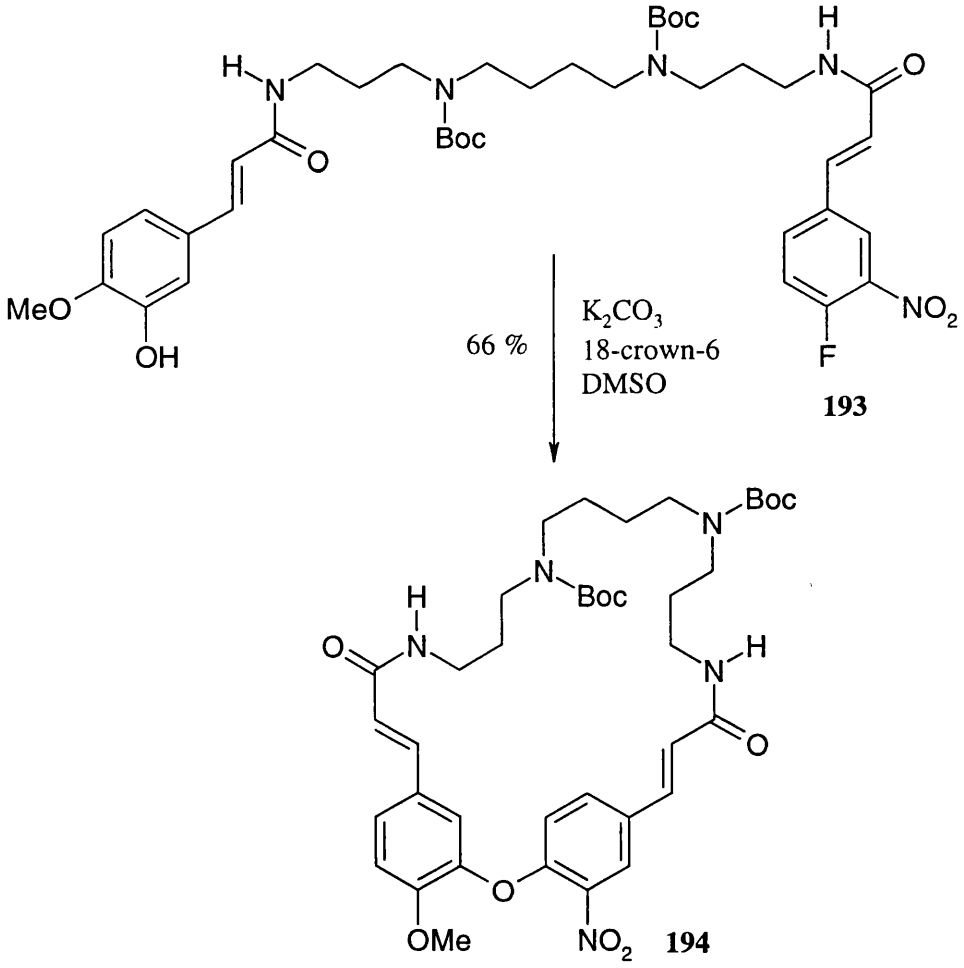


Fig. 5.15

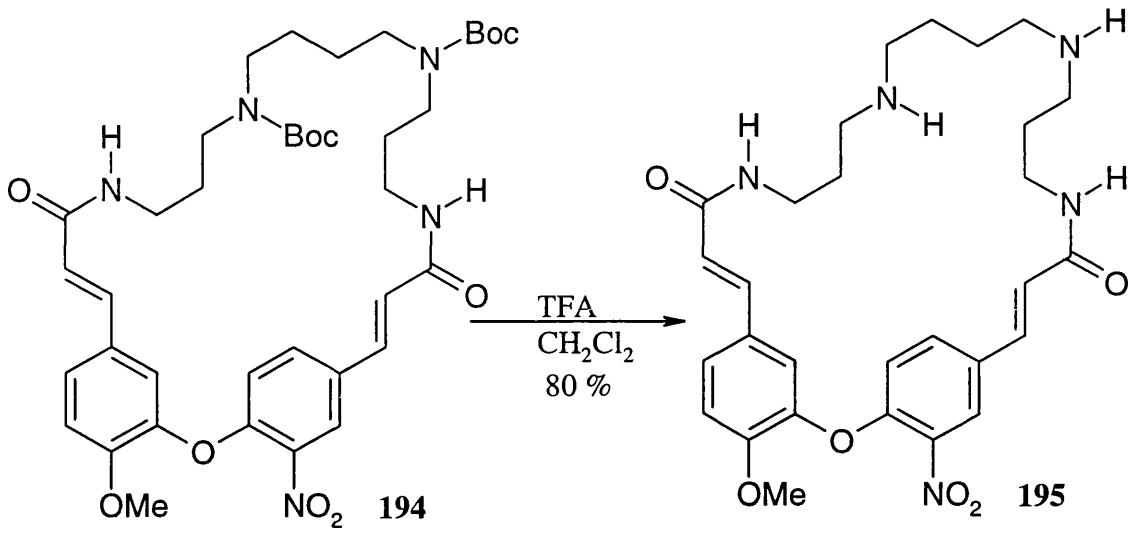


Fig. 5.16

Finally TFA in CH_2Cl_2 to strip off the Boc protecting groups and yield the diamine **195** after chromatography (Fig. 5.16).

The utility of S_NAr reactions has clearly been demonstrated with respect to the synthesis of polyamine macrocyclic alkaloids in a practical, high yielding fashion. It should find ready application in the synthesis of further compounds in the cadabicine and codonocarpine classes. For the first time, macrocyclic lactams of 23-, 24- and 28-members have been prepared. Rather than using intermolecular Ullmann chemistry to close the diphenyl ether bond, this was chosen as the site of disconnection for aromatic intramolecular nucleophilic substitution. We have prepared novel large-ring heterocycles for biological evaluation against the enzyme TR.

Chapter 6

Experimental

Experimental

General Details

All chemicals were purchased from the Sigma-Aldrich-Fluka Chemical company (Gillingham, Dorset) and used without further purification. All solvents were either GPR or HPLC grade purchased from BDH (Poole, Dorset) and were used without further distillation. Anhydrous solvents were prepared with reference to procedures described in "Purification of Laboratory Chemicals" (Perrin *et al.*, 1988). Water refers to singly distilled water. All solvent ratios are given as volume/volume. All reactions, unless otherwise stated, were performed under an atmosphere of anhydrous N_2 gas. When anhydrous reagents were used all glassware was first dried at120 °C, *in vacuo*. Thin layer chromatography (TLC) was performed routinely to monitor reaction progress and chemical purity. TLC was performed on pre-coated plates (Merck TLC aluminium sheets silica 60 F_{254}). Visualisation was with UV light (254 nm or 366 nm) and acidic ninhydrin reagent (0.3 g ninhydrin in 100 mL *n*-butanol and 3 mL acetic acid), alkaline potassium permanganate reagent (3 g potassium permanganate, 20 g potassium carbonate, 5 mL 5% aq. NaOH, 300 mL water) or iodine vapour as appropriate.

Flash column chromatography was performed according to the method of Still *et al.* (1978) using sorbasil C60-H silica gel purchased from Prolabo, (Eccles, Manchester) or ca. 150 mesh neutral alumina purchased from Sigma-Aldrich-Fluka. Columns were packed as a slurry in the eluting solvent and pressure was applied by hand bellows.

Reverse-phase HPLC was carried out using a 5 μm C8 inertpak column using a JASCO PU980 pump and a JASCO UV975 variable UV detector.

Melting points (MP) were determined using a Reichert-Jung Thermo Galen Kopfler block and are uncorrected. Infra-red spectra were recorded either as a thin

liquid film or as a KBr disc using a Perkin-Elmer 782 instrument. Ultra-violet spectra were obtained from solutions in methanol using a Perkin-Elmer Lambda 3 spectrometer. ^{1}H-NMR spectra were recorded at 270 or 400 MHz using either a Jeol GX270 or a Jeol EX400 spectrometer respectively. ^{13}C-NMR were recorded at either 67.8 (GX270) or 100.8 MHz (GX400). Assignments were assisted by the use of 135- and 90-DEPT carbon pulse experiments as well as ^{1}H-^{1}H and ^{1}H-^{13}C 2D correlation spectra. Chemical shifts (δ) are expressed in parts per million downfield from tetramethylsilane as internal standard. Coupling constants (*J*) are in Hertz and multiplicities are denoted by the abbreviations: s-singlet, bs-broad singlet, d-doublet, t-triplet, q-quartet, quin-quintet and m-multitplet. Assignments are listed as: δ (number of protons, multiplicity, coupling constant, assignment).

Mass spectra were obtained using a VG autospec spectrometer. Electron impact ionisation (EI) spectra were recorded typically using an ionising potential of 70 eV. Positive and negative FAB spectra were recorded using 3-nitrobenzyl alcohol as the matrix.

In vacuo refers to the use of a water aspirator, pressure (typically 15-30 mmHg). For the *in vacuo* removal of solvents a Büchi rotary evaporator was used and for drying solids *in vacuo* a vacuum desiccator was used with P_2O_5 as the drying agent unless otherwise stated.

Amide bond formation by DCC/HOBt condensation - General Procedure A.

The carboxylic acid (1 equiv.), the amine (1 equiv.), DCC (1.5 equiv.) and a catalytic amount of HOBt (0.05 equiv.) were dissolved in an organic solvent and stirred at 20 °C. The precipitate was filtered off and the filtrate was evaporated *in vacuo*. The residue was purified by flash column chromatography on silica gel to yield the desired amide.

Removal of Z-protecting groups by hydrogenolysis - General Procedure B.

A solution of Z-protected amine in MeOH was added to 10 % palladium on carbon. The mixture was hydrogenated at atmospheric pressure for 24 h and then filtered through celite. The filter cake was washed with MeOH (3 x 20 mL) and the combined filtrate was evaporated *in vacuo*. The residue was purified by flash column chromatography to yield the desired deprotected amine.

General method 1 for resin cleavage - General Procedure C.

The resin was swollen with CH_2Cl_2 and CH_2Cl_2/TFA (1:1) was added. The reaction mixture was stirred for 2 h at 20 °C and then the reaction mixture was filtered. The filter cake was washed well with CH_2Cl_2 and then MeOH. The combined filtrate and washes were evaporated *in vacuo*.

General method 2 for resin cleavage - General Procedure D.

The resin was swollen with CH_2Cl_2 and CH_2Cl_2/TFA (1:1) was added. The reaction mixture was stirred for 1 h at 0 °C and then the reaction mixture was filtered. The filter cake was washed well with CH_2Cl_2 and then MeOH. The combined filtrate and washes were evaporated *in vacuo*.

General method for azido reduction - General Procedure E.

Triphenylphosphine (340 mg per g of resin, 1.30 mmol) was dissolved in THF (5 mL) and water (10 drops). This solution was added to the resin and the reactor was sealed and agitated for 24 h. The resin was washed with THF (4 x 5 mL) and the procedure was repeated using the same quantities of reagents for the same length of time. The resin was washed successively with THF, THF/H_2O (1:1), THF and CH_2Cl_2 and was then dried *in vacuo* at 40°C over P_2O_5.

General method for acylation with HOBt activated 9- acridinecarboxylic acid - General Procedure F.

The resin (1 g) was mixed with the solution of HOBt activated 9- acridine carboxylic acid (1.3 mmol) in DMF (7 mL) then the reactor was flushed with N_2, sealed and agitated for 16 h. The resin was washed with anhydrous DMF (5 x 5 mL) and the procedure was repeated with the same quantity of reagent, agitating for 3 h. The resin was washed successively with DMF, THF, THF/H_2O (1:1), THF then Et_2O (5 x 5 mL each solvent) and was then dried *in vacuo* at 40°C over P_2O_5.

N^1-(*t*-Butoxycarbonyl)spermine 69

A solution of Boc anhydride (3.6 g, 17 mmol) in THF (30 mL) was added to a stirred solution of spermine **1** (10 g, 49 mmol) in THF (40 mL) at 0 °C. The mixture was stirred at this temperature for 1 h and then for a further 18 h at 20 °C. The solvent was removed *in vacuo* and then the residue was purified by flash column chromatography on silica gel eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (10:4:1, 6:4:1 then 4:2:1) to yield the title compound **69** as a colourless oil (2.28 g, 15 % with respect to spermine). ^{1}H-NMR ($CDCl_3$) δ 1.44 (9 H, s, 3 x C$\underline{H}_3$), 1.51-1.57 (4 H, m, 2 x $CH_2C\underline{H}_2CH_2$), 1.60-1.70

(4 H, m, 2 x $CH_2C\underline{H}_2CH_2$), 1.78, (4 H, bs, 2 x N$\underline{H}$, N$\underline{H}_2$), 2.58-2.68 (8 H, m, 2 x $C\underline{H}_2NHC\underline{H}_2$), 2.68-2.94 (2 H, m, $C\underline{H}_2NH_2$), 3.09-3.21 (2 H, m, $C\underline{H}_2NHCO$), 5.13 (1H, bs, N$\underline{H}$CO; ^{13}C NMR δ 27.7, 28.3 (3 x $\underline{C}H_3$), 29.9, 33.7, 39.0, 40.4, 47.5, 47.7, 49.7, 48.8, 78.9 ($\underline{C}(CH_3)_3$), 156.1 ($\underline{C}$=O); FAB-MS *m/z* 303 (MH^+), $C_{15}H_{34}N_4O_2$ requires 302.

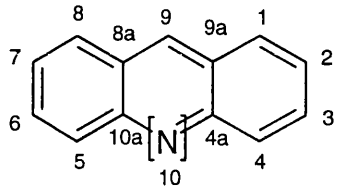
N^1-*t*-Butoxycarbonyl-N^2,N^3,N^4-(tribenzyloxycarbonyl)spermine 70

Benzyl chloroformate (2.7 mL, 18.8 mmol) was added dropwise (over 30 min) to a stirred solution of N^1-*t*-butoxycarbonylspermine **69** (1.725 g, 5.70 mmol) in aq. NaOH solution (2 M, 11 mL) at 0 °C. The mixture was stirred for 18 h at 20 °C then CH_2Cl_2 (20 mL) was added and the mixture stirred for a further 1 h. The organic layer was collected and the aq. layer was washed with CH_2Cl_2 (2 x 20 mL). The combined organic layers were dried ($MgSO_4$) and evaporated *in vacuo* to yield the title compound **70** as a colourless oil (4.24 g, 94 %) which was used without further purification. ^{1}H-NMR ($CDCl_3$) δ 1.30-1.57 (13 H, m, 3 x $C\underline{H}_3$, 2 x $CH_2C\underline{H}_2CH_2$), 1.57-1.74 (4 H, m, $CH_2C\underline{H}_2CH_2$), 2.90-2.30 (12 H, m, 1 x $C\underline{H}_2NHZ$, 4 x $C\underline{H}_2NZ$, 1 x $C\underline{H}_2NHBoc$), 5.07 (6 H, s, 3 x $C\underline{H}_2O$), 5.60-5.80 (2H, m, 2 x N$\underline{H}$C=O), 7.20-7.40 (15 H, m, 3 x Ph); FAB-MS *m/z* 705 (MH^+) $C_{39}H_{52}N_4O_8$ requires 704.

N^1,N^2,N^3-tri(Benzyloxycarbonyl)spermine 71

N^1-*t*-Butoxycarbonyl-N^2,N^3,N^4-(tribenzyloxycarbonyl)spermine **70** (4.00 g, 5.70 mmol) was stirred in trifluoroacetic acid (31 mL) and CH_2Cl_2 (30 mL) at 0 °C for 45 min. The solvents were removed *in vacuo*, as an azeotrope with water and MeOH. The residue was purified by flash column chromatography eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (200:10:1) to yield the title compound **71** as a colourless oil (3.38 g, 98 %). ^{1}H NMR δ ($CDCl_3$) 1.34-1.76 (10 H, m, 4 x $CH_2C\underline{H}_2CH_2$, N$\underline{H}_2$), 2.56-2.73 (2 H, m,

C$\underline{H}_2$NH$_2$), 3.00-3.40 (m, 10 H, 1 x C$\underline{H}_2$NHZ, 2 x C$\underline{H}_2$NZC$\underline{H}_2$), 5.10 (6 H, s, 3 x C$\underline{H}_2$O), 5.60-5.80 (1 H, m, CH$_2$N$\underline{H}$Z), 7.30 (15 H, bs, 3 x Ph); ^{13}C NMR δ 25.2, 31.6, 34.1, 44.0, 46.4, 46.7, 53.3, 66.4 (C$\underline{H}_2$O), 66.3 (C$\underline{H}_2$O), 67.1 (C$\underline{H}_2$O),127.8 (Ph), 127.9 (Ph), 128.4 (Ph), 136.5 (Ph) 156.1 (C=O), 156.4 (C=O); FAB-MS *m/z* 605 (MH$^+$), $C_{34}H_{44}N_4O_6$, requires 604.



N^1-(Anthracene-9-carbonyl)- N^2, N^3, N^4-tri(benzyloxycarbonyl)spermine 74

9-Anthracene carboxylic acid **72** (0.10 g, 0.45 mmol), N^1,N^2,N^3-tri(benzyloxycarbonyl)spermine **71** (0.272 g, 0.45 mmol), DCC (0.139 g, 0.68 mmol), HOBt (3 mg, 0.05 equiv.) and CH_2Cl_2 (5 mL) were used in general procedure A for amide bond formation. The reaction mixture was stirred for 18 h and was purified by flash column chromatography on silica gel eluting with 2 % MeOH/CH_2Cl_2 to yield the title compound **74** as a colourless oil (0.276 g, 76 %). ^{1}H NMR ($CDCl_3$) δ 1.35-1.70 (6 H, m, 3 x CH$_2$C$\underline{H}_2$CH$_2$), 1.72-2.04 (2 H, m, CH$_2$C$\underline{H}_2$CH$_2$), 2.92-3.73 (12 H, m, 1 x C$\underline{H}_2$NHZ, 2 x C$\underline{H}_2$NZC$\underline{H}_2$, 1 x C$\underline{H}_2$NHCOAr), 4.91 (2 H, s, C$\underline{H}_2$O), 5.01- (4 H, m, 2 x C$\underline{H}_2$O), 7.31 (15 H, m, 3 x Ph), 7.49-7.61 (4 H, m,C$\underline{H}^2$, C$\underline{H}^3$, C$\underline{H}^6$, C$\underline{H}^7$), 8.00-8.21 (4 H, m, C$^1\underline{H}$, C$^4\underline{H}$, C$^5\underline{H}$, C$^8\underline{H}$), 8.47-8.52 (1 H, m, C$^{10}\underline{H}$); ^{13}C-NMR δ 24.8, 25.9, 28.9, 30.3, 44.0, 46. 6, 49.1, 67.4 (C$\underline{H}_2$O), 68.35 (C$\underline{H}_2$O), 124.8, 125.7, 126.6, 127.5, 128.2, 128.4, 131.2, 151.8 (C^9), 169.7 (C=O); FAB-MS *m/z* 809 (MH$^+$), $C_{49}H_{51}N_4O_7$ requires 808

N^1-(Anthracene-9-carbonyl) spermine 67

A solution of N^1-(anthracene-9-carbonyl)- N^2, N^3, N^4-tri(benzyloxycarbonyl)spermine **74** (0.260 g, 0.32 mmol) in MeOH (50 mL) and 10 % palladium on carbon (18 mg) were used in general procedure B for hydrogenolysis. The mixture was hydrogenated for 24 h and the product was purified by flash column chromatography on silica gel eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (4:2:1) to yield the title compound **67** as a white, hygroscopic foam (0.122 g, 93 %). ^{1}H NMR (D_2O) δ 1.47 (4 H, bs, 2 x $CH_2C\underline{H}_2CH_2$), 1.58 (2H, t, *J*=7, $CH_2C\underline{H}_2CH_2$), 1.87, (2 H, t, *J*=7, $CH_2C\underline{H}_2CH_2$), 2.53-2.61 (6 H, m, 3 x $C\underline{H}_2NHCH_2$), 2.69 (2 H, t, *J*=7, $CH_2C\underline{H}_2CH_2$), 3.20 (2 H, bs, $C\underline{H}_2NH_2$), 3.57 (2 H, t, *J*=7, $C\underline{H}_2NHCOAr$), 7.40-7.48 (4 H, m, $C^2\underline{H}$, $C^3\underline{H}$, $C^6\underline{H}$, $C^7\underline{H}$), 7.91 (2 H, d, *J*=8, $C^4\underline{H}$, $C^5\underline{H}$), 7.98 (2 H, d, *J*=8 $C^1\underline{H}$, $C^8\underline{H}$), 8.49 (1 H, s, $C^{10}\underline{H}$); ^{13}C-NMR δ 24.6, 26.0, 29.8, 30.3, 43.8, 46. 6, 48. 6, 124.7, 125.7, 126.6, 127.5, 128.2, 131.2, 151.8 ($\underline{C}^9$), 169.7 ($\underline{C}$=O); UV λ_{max} = 247 nm, ε = 57000; FAB-MS *m/z* 407 (MH^+), $C_{25}H_{35}N_4O$ requires 406; FAB-HRMS calcd. 407.2811 (MH^+), found 407.2808; Found C 65.0, H 7.94, N 11.7, requires C 73.86, H 8.43, N 13.78 %.

N^1-(Acridine-9-carbonyl)- N^2, N^3, N^4-tri(benzyloxycarbonyl)spermine 75

9-Acridine carboxylic acid hydrate **73** (0.37 g, 1.66 mmol), N^1,N^2,N^3-tri(benzyloxycarbonyl)spermine **71** (1.0 g, 1.66 mmol), DCC (0.680 g, 3.30 mmol), HOBt (11 mg, 0.05 equiv.) and DMF (10 mL) were used in general procedure A for amide bond formation. The reaction mixture was stirred for 48 h and was purified by flash column chromatography on silica gel eluting with 2 % MeOH/CH_2Cl_2 to yield the title compound **75** as a colourless oil (0.707 g, 53 %). ^{1}H-NMR ($CDCl_3$) δ 1.33-1.70 (6 H, m, 3 x $CH_2C\underline{H}_2CH_2$), 1.77-2.03 (2 H, m, $CH_2C\underline{H}_2CH_2$), 2.93-3.33 (8 H, m, 4 x $C\underline{H}_2NZCH2$), 3.40 (2 H, bs, $C\underline{H}_2NHZ$), 3.62 (2 H, bs, $C\underline{H}_2NHCOAr$), 4.83-5.14 (6 H,

m, 3 x C$\underline{H}_2$O), 6.77 and 6.97 (2H, bd, N$\underline{H}$CO), 7.32 (15 H, m, 3 x Ph), 7.53 (2 H, t, *J*=8, C$^2\underline{H}$, C$^7\underline{H}$), 7.75 (2 H, t, *J*=8, C$^3\underline{H}$, C$^6\underline{H}$), 7.80-7.94 (1 H, m, C$^1\underline{H}$/C$^8\underline{H}$), 8.01 (1 H, d, *J*=9, C$^1\underline{H}$/C$^8\underline{H}$), 8.19 (2 H, d, *J*=9, C$^4\underline{H}$, C$^5\underline{H}$); ^{13}C-NMR δ 24.8, 25.5, 33.8, 44.0, 46.5, 66.4 (C$\underline{H}_2$O), 67.1 (C$\underline{H}_2$O), 122.2 (C^2, C^7), 125.2, 126.7, 127.7 (Ph), 127.8 (Ph), 128.0 (Ph), 128.4, 129.5 (C^4, C^5), 130.4 (C^3, C^6), 140. 2 (C^{10a}, C^{4a}), 148.4 (C^9), 167.0 (C=O); FAB-MS *m/z* 782 (MH$^+$), $C_{47}H_{52}N_5O_6$ requires 781.

N^1-(Acridine-9-carbonyl)spermine 68

A solution of N^1-(acridine-9-carbonyl)- N^2, N^3, N^4-tri(benzyloxycarbonyl)spermine **75** (0.70 g, 0.87 mmol) in MeOH (50 mL) was added to 10 % palladium on carbon (20 mg) and used in general procedure B for hydrogenolysis. The mixture was hydrogenated for 24 h and the product was purified by flash column chromatography on silica gel eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (4:2:1) to yield the title compound **68** as a buff coloured hygroscopic foam (0.324 g, 91 %). ^{1}H NMR (D_2O) δ 1.29-1.99 (8 H, m, 4 x $CH_2C\underline{H}_2CH_2$), 2.33-2.54 (2 H, m, C$\underline{H}_2$NHCH$_2$), 2.57-2.90 (8 H, m, 3 x C$\underline{H}_2$NHCH$_2$, 1 x C$\underline{H}_2$NH$_2$), 3.00-3.17 (2 H, m, C$\underline{H}_2$NHCOAr), 6.61-6.74 (4 H, m, C$^2\underline{H}$, C$^3\underline{H}$, C$^6\underline{H}$, C$^7\underline{H}$), 6.92-7.40 (4 H, m, C$^1\underline{H}$, C$^4\underline{H}$, C$^5\underline{H}$, C$^8\underline{H}$); ^{13}C-NMR δ 28.2, 30.2, 32.9, 38.2, 40.8, 115.7 (C^{8a}, C^{9a}), 119.3 (C^1, C^8), 121.4 (C^2, C^7), 129.6 (C^4, C^5), 130.0 (C^3, C^6), 141.4 (C^{10a}, C^{4a}), 148.3 (C^9), 176. 6 (C=O); UV λ_{max} = 250 nm, ε = 57000; $C_{24}H_{33}N_5O$, 407, FAB-MS *m/z* 408 (MH$^+$).

9-Chloroacridine 96 (El-Sherief *et al.*, 1983)

A suspension of 9-(10*H*)-acridone **95** (3.0 g, 15.4 mmol) in thionyl chloride (18 mL) and DMF (4 drops) was warmed to dissolve all the solid and then the solution was heated under reflux for a further 30 min. The solution was evaporated *in vacuo* and the

residue was suspended in CH_2Cl_2. This slurry was slowly (5 min) poured into crushed ice/conc. aq. NH_3 with vigorous swirling. The organic layer was separated and the aq. layer was washed with CH_2Cl_2 (2 x 30 mL). The combined organic layer was dried ($MgSO_4$) and then evaporated *in vacuo* to yield the title compound **96**, a pale yellow solid, MP 119-120 °C (lit. 118-120 °C, Ning *et al.*, 1976) (3.145 g, 96 %). ^{1}H-NMR ($CDCl_3$) δ 7.57 (2 H, t, *J*=5, $C^2\underline{H}$, $C^7\underline{H}$), 7.70 (2 H, t, *J*=5, $C^3\underline{H}$, $C^6\underline{H}$), 8.19 (2 H, d, *J*=9), 8.36 (2 H, d, *J*=9) ($\underline{C}^1$, $\underline{C}^8$ and $\underline{C}^4$, $\underline{C}^5$); ^{13}C-NMR δ 124.2, 124.5, 126.8, 129.7, 130.4, 148.8, EI-MS *m/z* 213 (M^+) $C_{13}H_8Cl^{35}N$ requires 213 and 215 (M^+) $C_{13}H_8Cl^{37}N$ requires 215.

9-Phenoxyacridine 97 (Dupré and Robinson, 1945)

NaOH (0.660 g, 16.5 mmol) was dissolved in phenol (14.41 g), stirred at 80 °C and then 9-chloroacridine **96** (2.869 g, 13.4 mmol) was added in one portion. The mixture was stirred at this temperature for 1.5 h, then poured into aq. NaOH solution (2 M, 20 mL) and this mixture was stirred for 18 h. The title compound **97**, a yellow precipitate (MP 124-125 °C, lit. 127-128 °C, Dupré and Robinson, 1945), was filtered off and dried *in vacuo* over phosphorus pentoxide (3.490 g, 99 %). ^{1}H-NMR ($CDCl_3$) δ 6.83 (2 H, d, *J*=8, $C^{2'}\underline{H}$, $C^{6'}\underline{H}$), 7.01 (1 H, t, *J*=7, $C^{4'}\underline{H}$), 7.23 (2 H, t, *J*=7, $C^{3'}\underline{H}$, $C^{5'}\underline{H}$), 7.41 (2 H, t, *J*=7, $C^2\underline{H}$, $C^7\underline{H}$), 7.74 (2 H, t, *J*=8, $C^3\underline{H}$,$C^6\underline{H}$), 8.07 (2 H, d, *J*=9, $C^1\underline{H}$, $C^8\underline{H}$), 8.27 (2 H, d, *J*=9, $C^4\underline{H}$, $C^5\underline{H}$); ^{13}C-NMR δ 115.7 ($\underline{C}^{2'}$, $\underline{C}^{6'}$), 122.8 ($\underline{C}^{4'}$ or $\underline{C}^1$, $\underline{C}^8$), 122.9 ($\underline{C}^1$, $\underline{C}^8$ or $\underline{C}^{4'}$), 126.1 ($\underline{C}^2$, $\underline{C}^7$), 129.7 ($\underline{C}^4$, $\underline{C}^5$), 130.2 ($\underline{C}3'$, $\underline{C}^{5'}$), 130.8 ($\underline{C}^3$,$\underline{C}^6$); FAB-MS *m/z* 272 (MH^+) $C_{19}H_{13}NO$ requires 271.

N^1-(Acridin-9-yl)-N^2, N^3, N^4-tri(benzyloxycarbonyl)spermine 99

A mixture of 9-phenoxyacridine **97** (0.9 g; 3.32 mmol) and N^1,N^2,N^3-tri(benzyloxycarbonyl)spermine **71** (2.0 g, 3.32 mmol) were mixed with phenol at 80 °C and then stirred at this temperature for 18 h. The reaction mixture was poured into CH_2Cl_2 (25 mL) and was washed with aq. NaOH (2 M, 3 x 20 mL). The combined aq. washes were extracted with CH_2Cl_2 (3 x 20 mL) and the combined organic layers were dried ($MgSO_4$) and then evaporated *in vacuo*. The residue was purified by flash column chromatography eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (200:10:1) to yield the title compound **99** as a yellow oil (1.173 g, 44 %) ^{1}H-NMR ($CDCl_3$) δ 1.40-1.75 (6 H, m, 3 x $CH_2C\underline{H}_2CH_2$), 1.84-2.07 (2 H, m, $CH_2C\underline{H}_2CH_2$), 3.05-3.32 (8 H, m, 4 x $C\underline{H}_2NZCH_2$), 3.43 (2 H, bs, $C\underline{H}_2NHZ$), 3.72 (2 H, bs, $C\underline{H}_2NHAr$), 5.08 (4 H, s, 2 x $C\underline{H}_2O$), 5.21 (2 H, s, $C\underline{H}_2O$), 5.69 (1 H, bs, $N\underline{H}CO$), 7.13-7.42 (17 H, m, 3 x Ph, $C\underline{H}^2$, $C\underline{H}^7$), 7.64 (2 H, t, *J*=5, $C\underline{H}^3$, $C\underline{H}^6$), 8.06 (2 H, d, *J*=6, $C\underline{H}^4$, $C\underline{H}^5$), 8.23 (2 H, d, *J*=6, $C\underline{H}^1$, $C\underline{H}^8$); ^{13}C-NMR δ 25.0, 25.6, 27.9,28.7, 29.2, 37.5, 38.1, 44.0, 46.2, 46.4, 46.7, 66.4 ($\underline{C}H_2O$), 67.1 ($\underline{C}H_2O$), 67.3 ($\underline{C}H_2O$), 116.9 ($\underline{C}^{4a}$, $\underline{C}^{9a}$), 122.8, 123.1 ($\underline{C}^1$, $\underline{C}^8$ and $\underline{C}^2$, $\underline{C}^7$), 127.8 (Ph), 127.9 (Ph), 128.5 (Ph), 129.1, 129.8 ($\underline{C}^3$, $\underline{C}^6$ and $\underline{C}^4$,$\underline{C}^5$), 136.4 (Ph), 149.1 ($\underline{C}^{4a}$, $\underline{C}^{10a}$), 151.9 ($\underline{C}^9$), 155.9 ($\underline{C}$=O), 156.4 ($\underline{C}$=O), 157.1 (C=O); FAB-MS *m/z* 782 (MH^+) $C_{47}H_{51}N_5O_6$ requires 781.

N^1-(Acridin-9-yl)spermine 89

A solution of N^1-(acridin-9-yl)-N^2, N^3, N^4-tri(benzyloxycarbonyl)spermine **99** (0.50 g, 0.64 mmol) in MeOH (50 mL) and 10 % palladium on carbon (20 mg) were used in general procedure B for hydrogenolysis. The mixture was hydrogenated for 24 h and the product was purified by flash column chromatography on neutral alumina (grade 4) eluting with MeOH/CH_2Cl_2 (20 %, 50 %, 100 %) to yield the title compound **89** as a

yellow hygroscopic foam (0.214 g, 88 %). ^{1}H-NMR (D_2O) δ 1.23-1.72 (8 H, m, 4 x $CH_2C\underline{H}_2CH_2$),1.90 (2 H, m, $C\underline{H}_2NHCH_2$), 2.38-2.79 (8 H, m, 3 x $C\underline{H}_2NHCH_2$, 1 x $C\underline{H}_2NH_2$), 3.15 (2 H, m, $C\underline{H}_2NHAcr$), 7.28 (2 H, m, $C\underline{H}^2$, $C\underline{H}^7$), 7.63 (2 H, m, $C\underline{H}^3$, $C\underline{H}^6$), 7.80 (2 H, m, $C\underline{H}^4$, $C\underline{H}^5$), 8.29 (2 H, m, $C\underline{H}^1$, $C\underline{H}^8$); ^{13}C-NMR δ 25.5, 26.4, 28.6, 39.3, 47.2, 47.5, 48.2, 49.4, 49.7, 49.8, 113.9, 120.6, 126.8, 137.9, 141.0 ($\underline{C}^{4a}$, $\underline{C}^{10a}$), 158.9 ($\underline{C}^9$); FAB-MS *m/z* 380 (MH^+), $C_{23}H_{33}N_5$ requires 379.

5-(*t*-Butoxycarbonylamino)pentanoic acid 78

A solution of Boc-anhydride (0.82 g, 3.76 mmol) in THF (7 mL) was added in a dropwise fashion over 10 min to a stirred suspension of 5-aminopentanoic acid **77** (0.40 g, 3.41 mmol) in THF (9 mL) at 0 °C. The mixture was stirred at this temperature for 1 h and then for a further 48 h at 20 °C (with TLC monitoring). The residue was taken up in CH_2Cl_2 and was washed with water (2 x 30 mL) and saturated aq. NH_4Cl (2 x 20 mL). The organic layer was dried ($MgSO_4$) and then concentrated *in vacuo*. The residue was purified by flash column chromatography eluting with CH_2Cl_2/MeOH/aq. NH_3 (15:5:1) to yield the title compound **78**, a colourless oil (0.602 g, 82 %). ^{1}H-NMR ($CDCl_3$) δ 1.35-1.71 (13 H, m, 3 x $C\underline{H}_3$, $C^3\underline{H}_2$, $C^4\underline{H}_2$), 2.23 (2 H, t, *J*=7, $C^2\underline{H}_2$), 3.07 (2 H, t, *J*=6, $C^5\underline{H}_2$), 5.28 (1 H, bs, N$\underline{H}$); ^{13}C-NMR 22.6 ($\underline{C}^3$), 28.3 (3 x $\underline{C}H_3$), 29.5 ($\underline{C}^4$), 35.4 ($\underline{C}^2$), 40.2 ($\underline{C}^5$), 79.0 ($\underline{C}(CH_3)_3$), 156.3 ($\underline{C}$ON), 179.1 ($\underline{C}$OOH); FAB-MS *m/z* 218 (MH^+), $C_{10}H_{19}NO_4$, requires 217.

N^1-[5-(*t*-Butoxycarbonylaminopentanoyl]-N^2, N^3, N^4-tri(benzyloxycarbonyl)-spermine 79

5-(*t*-Butoxycarbonylamino)pentanoic acid **78** (0.21 g, 0.97 mmol), N^1,N^2,N^3-tri(benzyloxycarbonyl)spermine **71** (0.585 g, 0.97 mmol), DCC (0.296 g, 1.43 mmol),

HOBt (6 mg) and CH_2Cl_2 (5 mL) were used in general procedure A for amide bond formation. The reaction mixture was stirred for 48 h and was purified by flash column chromatography eluting with CH_2Cl_2/MeOH/aq. NH_3 (200:10:1) to yield, a colourless oil **79** (0.30 g, 38 %). ^{1}H-NMR ($CDCl_3$) δ 1.42-1.89 (12 H, m, 6 x $CH_2C\underline{H}_2CH_2$), 1.53 (9 H, s, 3 x $C\underline{H}_3$), 3.12-3.47 (16 H, m, 7 x $C\underline{H}_2N$, $C\underline{H}_2C{=}O$), 4.91 (1H, bs, N$\underline{H}$CO), 5.22 (6 H, s, 3 x $C\underline{H}_2O$), 5.80 (1 H, bs, N$\underline{H}$CO), 6.77 (1 H, bs, N$\underline{H}$CO), 7.33-7.57 (15 H, m, Ph); ^{13}C-NMR δ 22.7, 25.2, 27.3, 28.2, 29.1, 33.6, 35.5, 37.1, 39.3, 44.1, 46.4, 53.0, 65.5 ($\underline{C}H_2O$), 66.8 ($\underline{C}H_2O$), 127.8 (Ph), 127.9 (Ph), 128.4 (Ph), 128.4 (Ph), 136.5 (Ph), 155.9 (C=O), 156.4 (C=O), 156.6 (C=O), 172.7 (C=ON); FAB-MS *m/z* 804 (MH^+), $C_{44}H_{61}N_5O_9$, requires 803.

N^1-[5-Aminopentanoyl]-N^2, N^3, N^4-tri(benzyloxycarbonyl) spermine 80

N^1-[5-(*t*-butoxycarbonylaminopentanoyl]-N^2, N^3, N^4-tri(benzyloxycarbonyl)spermine **79** (0.30 g, 0.37 mmol) was stirred in trifluoroacetic acid (2 mL) and CH_2Cl_2 (2 mL) at 0 °C for 45 min. The solvents were removed *in vacuo*, as an azeotrope with water and MeOH. The residue was purified by flash column chromatography eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (100:10:1) to yield the title compound **80** as a colourless oil (0.230 g, 88 %). ^{1}H-NMR ($CDCl_3$) δ 1.34-1.78 (12 H, m, 6 x $CH_2C\underline{H}_2CH_2$), 2.24 (2 H, bs, $C\underline{H}_2NH_2$), 2.95 (2 H, bs, $C\underline{H}_2C{=}O$), 3.00-3.32 (12 H, m, 2 x $C\underline{H}_2NZC\underline{H}_2$, 2 x $C\underline{H}_2NHC{=}O$), 5.09 (6 H, s, $C\underline{H}_2O$), 7.23-7.34 (15 H, m, 3x Ph); ^{13}C-NMR δ 22.65, 24.7, 25.6, 27.0, 27.7, 28.4, 28.8, 32.0, 35.7, 36.1, 37.3, 41.2, 43.9, 46.2, 65.9 ($\underline{C}H_2O$) , 66.8 ($\underline{C}H_2O$), 126.7 (Ph), 127.4 (Ph), 136.2 (Ph), 155.9 (C=O), 156.4 (C=O), 157.1 (C=O), 172.9 (C=O); FAB-MS *m/z* 704 (MH^+) $C_{39}H_{53}N_5O_7$ requires 703.

N^1-(5-(Acridin-9-ylcarbonyl amino)pentanoyl)-N^2, N^3, N^4-tri(benzyloxycarbonyl)-spermine 81

9-Acridine carboxylic acid hydrate **73** (0.145 g, 0.65 mmol), N^1-[5-aminopentanoylamido]-N^2, N^3, N^4-tri(benzyloxycarbonyl)spermine **80** (0.456 g, 0.65 mmol), DCC (0.20 g,0.97 mmol), HOBt (4 mg, 0.05 equiv.) and DMF (5 mL) were used in general procedure A for amide bond formation. The reaction mixture was stirred for 48 h and was purified by flash column chromatography on silica gel eluting with 2 % MeOH/CH_2Cl_2 to yield the title compound **81** as a pale yellow oil (0.180 g, 31 %). ^{1}H-NMR ($CDCl_3$) δ 1.29-1.75 (12 H, m, 6 x $CH_2C\underline{H}_2CH_2$), 2.98-3.35, (14 H, m, 7 x $C\underline{H}_2N$), 3.65 (2 H, m, $C\underline{H}_2C{=}O$), 4.95 (6 H, m, 3 x $C\underline{H}_2O$), 7.28 (15 H, m, 3 x Ph), 7.50 (2 H, t, *J*=7, $C\underline{H}^2$, $C\underline{H}^7$), 7.73 (2 H, t, *J*=7, $C\underline{H}^3$, $C\underline{H}^6$), 7.96 (2 H, d, *J*=9, $C\underline{H}^4$, $C\underline{H}^5$), 8.17 (2 H, d. *J*=9, $C\underline{H}^1$, $C\underline{H}^8$); ^{13}C-NMR δ 22.3, 25.5, 30.9, 33.7, 35.5, 44.0, 46.0, 46.5, 50.0, 66.3 ($\underline{C}H_2O$), 67.4 ($\underline{C}H_2O$), 116.4, 122.8, 127.6 (Ph), 127.8 (Ph), 128.5, 136. 5, 148.8 ($\underline{C}^9$), 156.0 ($\underline{C}{=}O$), 156.5 ($\underline{C}{=}O$), 172.9 ($\underline{C}{=}ON$); FAB-MS *m/z* 909 (MH^+), $C_{53}H_{60}N_6O_8$ requires 908.

N^1-(5-(Acridin-9-ylcarbonylamino)pentanoyl)spermine 76

A solution of N^1-(5-(acridin-9-ylcarbonylamino)pentanoyl)-N^2, N^3, N^4-tri(benzyloxycarbonyl)spermine **81** (150 mg, 0.17 mmol) in MeOH (20 mL) was added to 10 % palladium on carbon (20 mg) and used in general procedure B for hydrogenolysis. The mixture was hydrogenated for 24 h and the product was purified by flash column chromatography on neutral alumina (grade 4) eluting with 20 % MeOH/CH_2Cl_2 to yield the title compound **76** as a buff coloured hygroscopic foam (0.067 g, 80 %). ^{1}H-NMR ($CDCl_3$) δ 1.22-1.69 (12 H, m, 6 x $CH_2C\underline{H}_2CH_2$), 2.75-3.02 (14 H, m, 7 x $C\underline{H}_2N$), 3.63 (2 H, m, $C\underline{H}_2C{=}O$), 6.97 (2 H, t, *J*=7, $C\underline{H}^2$, $C\underline{H}^7$), 7.10 (2 H,

t, *J*=7, C$\underline{H}^3$, C$\underline{H}^6$), 7.58 (2 H, d, *J*=9, C$\underline{H}^4$, C$\underline{H}^5$), 7.71 (2 H, d. *J*=9, C$\underline{H}^1$, C$\underline{H}^8$); FAB-MS *m/z* 505 (MH^+) $C_{29}H_{42}N_6O_2$ requires 506.

N^1-(5-(Acridin-9-ylamino)pentanoyl)-N^2, N^3,N^4-tri(benzyloxycarbonyl)spermine 100

A mixture of 9-phenoxyacridine **97** (0.089 g, 0.33 mmol) and N^1-[5-aminopentanoylamido]-N^2, N^3, N^4-tri(benzyloxycarbonyl)spermine **80** (0.23 g, 0.33 mmol) were mixed with phenol at 80 °C and then stirred at this temperature for 18 h. The reaction mixture was poured into CH_2Cl_2 and was washed with aq. NaOH (2 M, 3 x 20 mL). The combined aq. washes were extracted with CH_2Cl_2 (3 x20 mL) and the combined organic layers were dried ($MgSO_4$) and then evaporated *in vacuo*. The residue was purified by flash column chromatography eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (200:10:1) to yield the title compound **100** as a yellow oil (0.144 g, 49 %). ^{1}H-NMR ($CDCl_3$) δ 1.40 (4 H, bs, $CH_2C\underline{H}_2CH_2$), 1.60 (6 H, bs $CH_2C\underline{H}_2CH_2$), 1.78 (2 H, bs, $CH_2C\underline{H}_2CH_2$), 3.00-3.33 (14 H, m, 7 x C$\underline{H}_2$N), 3.78 (2 H, bs, C$\underline{H}_2$C=O), 5.03 (6 H, s, 3 x C$\underline{H}_2$O), 6.01 (1 H, bs, N$\underline{H}$CO), 6.90)1 H, bs, N$\underline{H}$CO), 7.11-7.32, (17 H, m, C$^2\underline{H}$, C$^7\underline{H}$, 3 x Ph), 7.56 (2 H, t, *J*=7, C$^3\underline{H}$, C$^6\underline{H}$), 7.97 (2 H, d, *J*=9, C$^4\underline{H}$, C$^5\underline{H}$), 8.14 (2 H, d, *J*=9, C$^1\underline{H}$, C$^8\underline{H}$); ^{13}C-NMR δ 22.0, 22.2, 22.7, 24.9, 25.5, 27.4, 27.9, 28.7, 29.5, 30.2, 32.7, 35.4, 35.6, 36.2, 37.5, 38.0, 41.4, 43.9, 46.2, 46.6, 49.7, 66.3 (C$\underline{H}_2$O), 67.0 (C$\underline{H}_2$O), 115.4, 116.9, 120.8, 120.9, 122.7, 123.3, 126.5, 127.6 (Ph), 127.8 (Ph), 128.2 (Ph), 128.3 (Ph), 130.6, 132.9, 136.3, 149.8, 152.7 (C^9), 155.9 (C=O), 156.4 (C=O), 156.7 (C=O), 172.7 (NC=O); FAB-MS *m/z* 881 (MH^+), $C_{52}H_{61}N_6O_7$ requires 880; FAB-HRMS calcd. 881.4602 (MH^+), found 881.4601.

N^1-(5-(Acridin-9-yl-amino)pentanoyl)spermine 90

A solution of N^1-(5-(acridin-9-ylamino)pentanoyl)-N^2, N^3,N^4-tri(benzyloxycarbonyl)spermine **100** (120 mg, 0.14 mmol) in MeOH (20 mL) was added to 10 % palladium on carbon (20 mg) and used in the general method for hydrogenolysis. The mixture was hydrogenated for 24 h and the product was purified by flash column chromatography on neutral alumina (grade 4) eluting with 20 % MeOH/CH_2Cl_2 to yield the title compound **90** as a hygroscopic yellow foam (53 mg, 79 %). ^{1}H-NMR (D_2O) δ 1.63-2.09 (12 H, m, 6 x $CH_2C\underline{H}_2CH_2$), 2.42-2.51 (2 H, m, $C\underline{H}_2NH_2$), 2.88-3.20 (12 H, m, 3 x $C\underline{H}_2NHC\underline{H}_2$), 4.08-4.12 (2 H, m, $CH_2C{=}O$), 7.48-7.51 (2 H, m, $C\underline{H}^2$, $C\underline{H}^7$), 7.67-7.75 (2 H, m, $C\underline{H}^3$, $C\underline{H}^6$), 7.88-7.93 (2 H, m, $C\underline{H}^4$, $C\underline{H}^5$), 8.29-8.35 (2 H, m, $C\underline{H}^1$, $C\underline{H}^8$); ^{13}C-NMR δ 25.0, 27.9, 29.2, 29.3, 32.1, 34.8, 40.2, 42.4, 118.1, 119.1, 126.3, 126.4, 127.4, 127.5, 128.2, 129.9, 131.7, 134.4, 141.5, 147.8, 173.1; FAB-MS *m/z* 479 (MH^+) $C_{28}H_{42}N_6O$ requires 478; Found C 46.2, H 5.38, N 8.72, requires C 45.66, H 4.82, N 9.13 %.

Methyl 5-aminopentanoate, HCl salt 101 (Kukla *et al.*, 1990)

Conc. aq. H_2SO_4 (50 mL, 96 %) was dropped onto solid NaCl (50 g) and the resulting HCl gas was bubbled through MeOH (60 mL) until gas production ceased. 5-Aminopentanoic acid **77** (6.50 g, 55.5 mmol) was added to the acidic MeOH and the solution was heated under reflux for 3 h. The reaction mixture was evaporated *in vacuo* and the residue was recrystallised (MeOH) to yield the title compound **101** as a white solid MP 140-141 °C (Lit. 145-146 °C, Oelofsen and Li, 1968) (8.42 g, 91 %). ^{1}H-NMR ($CDCl_3$) δ 1.67-1.90 (4 H, m, $CH_2C\underline{H}_2C\underline{H}_2CH_2$), 2.20 (2 H, t, *J*=7, $C\underline{H}_2NH_3^+$), 3.06 (2 H, bs, $C\underline{H}_2C{=}O$), 3.68 (s, 3 H, $C\underline{H}_3$).

Methyl 5-(5-(*t*-butoxycarbonylamino)pentanoylamino)pentanoate 102

5-(*t*-Butoxycarbonylamino)pentanoic acid **78** (0.30 g, 1.38 mmol), methyl 5-aminopentanoate **101** (0.181 g, 1.38 mmol), DCC (0.433 g, 2.10 mmol), HOBt (9 mg, 0.05 equiv.) and CH_2Cl_2 (5 mL) were used in general procedure A for amide bond formation. The reaction mixture was stirred for 48 h and was purified by flash column chromatography on silica gel eluting with 2 % MeOH/CH_2Cl_2 to yield the title compound **102** as a colourless oil (0.206 g, 45 %). ^{1}H-NMR ($CDCl_3$) δ 1.38-1.55 (17 H, m, 3 x C$\underline{H}_3$, 4 x $CH_2C\underline{H}_2CH_2$), 2.25-2.34 (2 H, m), 3.05-3.35 (6 H, m), 4.10 (3 H, s, COC$\underline{H}_3$); ^{13}C-NMR δ ; FAB-MS *m/z* 331 (MH^+), $C_{16}H_{30}N_2O_5$ requires 330.

5-(5-(*t*-Butoxycarbonylamino)pentanoylamino)pentanoic acid 103

Methyl 5-(5-(*t*-butoxycarbonylamino)pentanoylamino)pentanoate **102** (0.2 g, 0.61 mmol) was stirred in dioxane (5 mL) and aq. potassium hydroxide solution (2 M, 5 mL) for 2 h. The reaction mixture was neutralised (aq. 2 M HCl) and dioxane removed *in vacuo*. The remaining aq. suspension was extracted with CH_2Cl_2 (3 x 20 mL) and the combined organic layers were dried ($MgSO_4$) and evaporated *in vacuo*. The residue was purified by flash column chromatography eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (200:10:1) to yield the title compound **103** as a colourless oil (0.174 g, 90 %). ^{1}H-NMR ($CDCl_3$) δ 1.35-1.50 (17 H, m, 3 x C$\underline{H}_3$, 4 x $CH_2C\underline{H}_2CH_2$), 2.21 (2 H, m), 3.01-3.41 (6 H, m, 2 x C$\underline{H}_2$N, C$\underline{H}_2$CO); FAB-MS *m/z* 317 (MH^+), $C_{15}H_{28}N_2O_5$ requires 316.

***N*^{1}-5-(5-(*t*-Butoxycarbonylamino)pentanoylamino)pentanoyl-*N*^{2}, *N*^{3}, *N*^{4}-tri(benzyloxycarbonyl)spermine 104**

5-(5-(*t*-Butoxycarbonylamino)pentanoylamino)pentanoic acid **103** (0.30 g, 0.95 mmol), *N*^{1},*N*^{2},*N*^{3}-tri(benzyloxycarbonyl)spermine **71** (0.574 g, 0.95 mmol), DCC (0.293 g, 1.42

mmol), HOBt (9 mg, 0.05 equiv.) and CH_2Cl_2 (15 mL) were used in general procedure A for amide bond formation. The reaction mixture was stirred for 48 h and was purified by flash column chromatography on silica gel eluting with 2 % MeOH/CH_2Cl_2 to yield the title compound **104** as a colourless oil (0.311 g, 36 %). ^{1}H-NMR ($CDCl_3$) δ 1.33-1.90 (27 H, m,), 3.06-3.51 (18 H, m), 5.26 (6 H, s, 3 x OC$\underline{H}_2$), 7.35-7.41 (15 H, m, 3 x Ph); ^{13}C-NMR δ 22.65, 25.2, 27.3, 28.0 (3 x $\underline{C}H_3$), 34.0, 35.4, 37.0, 39. 6, 44.1, 46.4, 52.9, 65.4 (O$\underline{C}H_2$), 66.8 (O$\underline{C}H_2$), 78.8 ($\underline{C}(CH_3)_3$), 127.6 (Ph), 127.6 (Ph), 128.3 (Ph), 128.4 (Ph), 136.4 (Ph), 156.2 (C=O), 172.7 (C=O), 173.0 (C=O); FAB-MS *m/z* 903 (MH^+), $C_{49}H_{70}N_6O_{10}$ requires 902.

N^1-5-(5-Aminopentanoylamino)pentanoyl-N^2, N^3, N^4-tri(benzyloxycarbonyl)-spermine 105

N^1-5-(5-(*t*-Butoxycarbonylamino)pentanoylamino)pentanoyl-N^2, N^3, N^4-tri(benzyloxycarbonyl)spermine **104** (0.30 g, 0.33 mmol) was stirred in trifluoroacetic acid (2 mL) and CH_2Cl_2 (2 mL) at 0 °C for 45 min. The solvents were removed *in vacuo*, as an azeotrope with water and MeOH. The residue was purified by flash column chromatography eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (200:10:1) to yield the title compound **105** as a colourless oil (0.263 g, 99 %). ^{1}H-NMR ($CDCl_3$) δ 1.29-1.72 (16 H, m, 8 x $CH_2C\underline{H}_2CH_2$), 2.54 (2 H, m, $C\underline{H}_2NH_2$), 2.65-2.68 (4 H, m, 2 x $C\underline{H}_2C$=O), 3.04-3.36 (14 H, m, 2 x $C\underline{H}_2NZC\underline{H}_2$, 2 x $C\underline{H}_2NHC$=O, $C\underline{H}_2NZ$), 5.20 (6 H, s), 7.30 (15 H, m); ^{13}C-NMR δ 22.5, 24.6, 25.7, 27.1, 27.7, 28.3, 29.9, 23. 6, 35.7, 36.6, 37.3, 42.18, 43.9, 44.0, 46.2, 65.9, 67.1, 112.8, 113.0, 139.3, 156. 9, 157.0, 157.2, 172. 9, 173.0;FAB-MS *m/z* 803 (MH^+), $C_{44}H_{62}N_6O_8$ requires 802.

N^1-5-(5-(Acridin-9-ylamino)pentanoylamino)pentanoyl-N^2, N^3, N^4-tri(benzyloxycarbonyl)spermine 106

N^1-5-(5-Aminopentanoylamino)pentanoyl-N^2, N^3, N^4-tri(benzyloxycarbonyl)spermine **105** (0.25 g, 0.31 mmol) and 9-phenoxyacridine **97** (0.084 g; 0.31 mmol) were mixed with phenol (1.0 g) at 80 °C and were stirred for 18 h. The reaction mixture was poured into CH_2Cl_2 and was washed with aq. NaOH (2 M, 3 x 20 mL). The combined aq. washes were extracted with CH_2Cl_2 and the combined organic layers were dried ($MgSO_4$) and then evaporated *in vacuo*. The residue was purified by flash column chromatography eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (200:10:1) to yield the title compound **106** as a yellow oil (0.127 g, 45 %). ^{1}H-NMR ($CDCl_3$) δ 1.39-1.80 (16 H, m, 8 x $CH_2\underline{CH_2}CH_2$), 2.98-3.39, (16 H, m, 8 x $C\underline{H_2}N$), 3.80 (4 H, m, 2 x $C\underline{H_2}C{=}O$), 5.10 (6 H, s, 3 x $C\underline{H_2}O$), 7.30 (17 H, m), 7.54 (2 H, t, *J*=7), 7.95 (2 H, d, *J*=9), 8.14 (2 H, d, *J*=9); FAB-MS *m/z* 980, $C_{57}H_{69}N_7O_8$ requires 979.

N^1-5-(5-(Acridin-9-ylamino)pentanoylamino)pentanoylspermine 91

A solution of N^1-5-(5-(acridine-9-ylamino)pentanoylamino)pentanoyl-N^2, N^3, N^4-tri(benzyloxycarbonyl)spermine **106** (0.10 g, 0.10 mmol) in MeOH (20 mL) was added to 10 % palladium on carbon (20 mg) and used in general procedure B for hydrogenolysis. The mixture was hydrogenated for 24 h and the product was purified by flash column chromatography on neutral alumina (grade 4) eluting with MeOH/CH_2Cl_2 (20%, 50%, 100%) to yield the title compound **91** as a yellow hygroscopic foam (0.048 g, 81 %). ^{1}H-NMR ($CDCl_3$) δ 1.35-1.78 (16 H, m), 2.75-3.05 (16 H, m), 3.89 (4 H, m), 7.39 (2 H, m), 7.62 (2 H, m), 7.83 (2 H, m) 8.19, (2 H, m); ^{13}C-NMR δ 24.9, 27.9, 27.9, 29.2, 29.3, 29.4, 32. 6, 35.7, 42. 6, 42.6, 116.7, 120.5,

122.6, 130.6, 131.1, 142.5, 148.1, 172.7, 172.8; FAB-MS *m/z* 578 (MH^+), $C_{33}H_{51}N_7O_2$ requires 577.

Methyl 4-aminobenzoate 111 (Hosangadi and Dave, 1996)

Thionyl chloride (1.3 mL, 17.8 mmol) was added in a dropwise fashion (15 min) to MeOH (32.5 mL) being stirred at 0 °C. 4-Aminobenzoic acid **109** (0.50 g, 3.56 mmol) was added to the solution and the mixture was stirred for 18 h at 20 °C. The reaction mixture was neutralised with aq. NaOH (1 M) and the MeOH was removed *in vacuo*. The remaining aq. suspension was extracted with CH_2Cl_2 (3 x 30 mL) and the combined organic extracts were dried (Na_2SO_4) and evaporated *in vacuo*. The residue was recrystallised (MeOH) to yield the title compound **111** as a white solid (0.447 g, 82 %). MP 108-109 °C (Lit. 108-110 °C, Carr, 1972); ^{1}H-NMR ($CDCl_3$) δ 3.86 (3 H, s, $OC\underline{H}_3$), 4.15 (2 H, bs, $N\underline{H}_2$) 6.63 (2 H, d, *J*=5, $C^3\underline{H}$, $C^5\underline{H}$), 7.83 (2 H, d, *J*=5, $C^2\underline{H}$, $C^6\underline{H}$); ^{13}C-NMR δ 51.8 ($O\underline{C}H_3$), 113.6 ($\underline{C}^3$, $\underline{C}^5$), 119.1 ($\underline{C}^1$), 131.6 ($\underline{C}^2$, $\underline{C}^6$), 150.1 ($\underline{C}^4$), 167.3 (C=O); EI-MS *m/z* 151 (M^+), $C_8H_9NO_2$ requires 151.

Methyl 4-(acridin-9-ylamino)benzoate 113

9-Chloroacridine **96** (0.555 g, 2.60 mmol) and methyl 4-aminobenzoate **111** (0.393 g, 2.60 mmol) were stirred in anhydrous MeOH (10 mL) until all the solid had dissolved. Methanesulfonic acid (0.3 mL) was added and the reaction mixture was stirred for 18 h. The mixture was neutralised with aq. NaOH solution (2 M) and was evaporated *in vacuo*. The residue was partitioned between CH_2Cl_2 and water and the aq. layer was washed with CH_2Cl_2 (2 x 40 mL). The combined organic layers were dried (Na_2SO_4) and then evaporated *in vacuo*. The residue was purified by flash column

chromatography eluting with 5 % ethyl acetate-hexane to yield the title compound **113** as an orange solid (0.769 g, 85 %). MP 169-170 °C; ^{1}H-NMR ($CDCl_3$) δ 3.87 (3 H, s, $OC\underline{H}_3$), 6.80 (2 H, t, *J*=9, $C\underline{H}^{3'}$, $C\underline{H}^{5'}$), 7.15-7.28 (2 H, m, $C\underline{H}^2$, $C\underline{H}^7$), 7.59 (2 H, t, *J*=7, $C\underline{H}^3$, $C\underline{H}^6$), 7.82 (2 H, bs, $C\underline{H}^{2'}$, $C\underline{H}^{6'}$), 7.93 (2H, d, *J*=9, $C\underline{H}^4$, $C\underline{H}^5$), 7.98 (2H, d, *J*=9, $C\underline{H}^1$, $C\underline{H}^8$); ^{13}C-NMR δ 51.8 ($O\underline{C}H_3$), 116.1, 120.1, 122.1, 123.8, 125.3, 130.8, 131.5, 167.1 (C=O); FAB-MS *m/z* 329 (MH^+) $C_{21}H_{16}N_2O_2$ requires 328.

4-(Acridin-9-ylamino)benzoic acid 115

Methyl 4-(acridin-9-ylamino)benzoate **113** (0.70 g, 2.13 mmol) was stirred in dioxane (20 mL) and aq. KOH solution (2 M, 20 mL) for 6 h. The reaction mixture was neutralised (aq. 2 M HCl) and dioxane removed *in vacuo*. The remaining aq. suspension was filtered and the filter cake was purified by flash column chromatography eluting with 10 % ethyl acetate-hexane to yield the title compound **115** as an orange solid (0.570 g, 85 %). MP 285-287 °C; ^{1}H-NMR (CD_3OD) δ 7.03 (2H, d, *J*=9, $C\underline{H}^{3'}$, $C\underline{H}^{5'}$), 7.16 (2 H, t, *J*=7, $C\underline{H}^2$, $C\underline{H}^7$), 7.63-7.70 (4 H, m, $C\underline{H}^{2'}$, $C\underline{H}^{6'}$, $C\underline{H}^3$, $C\underline{H}^6$), 7.91 (2 H, d, *J*=9, $C\underline{H}^4$, $C\underline{H}^5$), 7.98 (2 H, d, *J*=9, $C\underline{H}^1$, $C\underline{H}^8$); ^{13}C-NMR δ 117.2, 119.4, 119. 7, 122.4, 124.7, 126.5, 131.3, 132.9, 141.5, 151.2, 167.2 ($\underline{C}$=O); $C_{20}H_{14}N_2O_2$ FAB-MS *m/z* 315 (MH^+) $C_{20}H_{14}N_2O_2$ requires 314.

***N*^{1}-[4'-(Acridin-9-ylamino)benzoyl]-*N*^{2},*N*^{3},*N*^{4}-tri(benzyloxycarbonyl) spermine 117**

4'-(Acridin-9-ylamino)benzoic acid **115** (0.325 g, 1.03 mmol), *N*^{1},*N*^{2},*N*^{3}-tri(benzyloxycarbonyl)spermine **71** (0.625 g, 1.03 mmol), DCC (0.320 g, 1.55 mmol) and HOBt (7 mg) were combined in THF (7 mL) according to general procedure A for amide bond formation. After stirring for 48 h the mixture was filtered and the filtrate

was evaporated *in vacuo*. The residue was purified by flash column chromatography eluting with 10 % MeOH-CH_2Cl_2 to yield an orange oil **117** (0.282 g, 31 %). ^{1}H-NMR ($CDCl_3$) δ 1.35-1.85 (8 H, m, 4 x $CH_2C\underline{H}_2CH_2$), 2.98-3.40 (12 H, m, 6 x $C\underline{H}_2N$), 5.10 (6 H, s, 3 x $C\underline{H}_2O$), 7.19 (2 H. t, *J*=7, $C\underline{H}^2$, $C\underline{H}^7$), 7.22-7.49 (17 H, m), 7.61 (2 H, t, *J*=7, $C\underline{H}^3$, $C\underline{H}^6$), (7.95, 6 H, m); ^{13}C-NMR δ 25.6, 27.9, 32.2, 33.8, 43.8, 46.1, 66.0, 67.4, 116. 7, 121.0, 124.2, 125.20, 125.6, 127.4, 128.3, 133.8, 136.5, 141.1 ,152.1, 156.2, 157.1; FAB-MS *m/z* 901 (MH^+), $C_{54}H_{56}N_6O_7$ requires 900.

N^1-[4'-(Acridin-9-ylamino)benzoyl]spermine 107

A solution of N^1-[4'-(acridin-9-ylamino)benzoyl]-N^2,N^3,N^4-tri(benzyloxycarbonyl) spermine **117** (0.50 g, 0.55 mmol) in MeOH (50 mL) and 10 % palladium on carbon (20 mg) were used in general procedure B for hydrogenolysis. The mixture was hydrogenated for 24 h and the product was purified by flash column chromatography on neutral alumina (grade 4) eluting with MeOH-CH_2Cl_2 (20%, 50%, 100%) to yield the title compound **107** as an orange hygroscopic foam (0.172 g, 61 %). ^{1}H-NMR (D_2O) δ 1.25-2.01 (8 H, m, 4 x $CH_2C\underline{H}_2CH_2$), 2.35 (2 H, m, $C\underline{H}_2NH_2$), 2.51-2.85 (8 H, m, 2 x $C\underline{H}_2NHC\underline{H}_2$), 3.15 (2 H, m. $C\underline{H}_2CONAr$), 7.21 (2 H, t, *J*=7, $C^2\underline{H}$, $C^7\underline{H}$), 7.34 (2 H, d, *J*=8), 7.42 (2 H, d, *J*=8), 7.55 (2 H, t, *J*=7), 7.89-7.94 (4 H, m); ^{13}C-NMR δ 28.5, 29.1, 30.2, 23.9, 38.2, 41.1, 42.7, 116.8, 119.3, 120.1, 122.3, 125.4, 137.0, 132.5, 133.8, 139.8, 141.1, 122.3, 157.2 ($\underline{C}$=O); FAB-MS *m/z* 499 (MH^+), $C_{30}H_{38}N_6O$ requires 498.

Methyl 4-amino-3-methoxybenzoate 112

Thionyl chloride (0.8 mL, 11.0 mmol) was added in a dropwise fashion (15 min) to MeOH (20 mL) stirred at 0 °C. 4-Amino-3-methoxybenzoic acid **110** (0.40 g, 0.60

mmol) was added to the solution and the mixture was stirred for 18 h at 25 °C. The reaction mixture was neutralised with aq. NaOH solution (1 M) and MeOH was removed *in vacuo*. The remaining aq. suspension was extracted with CH_2Cl_2 (3 x 20 mL) and the combined organic extracts were dried (Na_2SO_4) and evaporated *in vacuo*. The residue was recrystallised (MeOH) to yield the title compound **112** (0.337 g, 78 %) as a white solid, MP 125-127 °C (Lit. 127-128 °C, Rizzacasa and Sargent, 1988). ^{1}H NMR ($CDCl_3$) δ 3.85 (3 H, s, $C\underline{H}_3$), 3.88, (3 H, s, $C\underline{H}_3$), 4.28 (2 H, s, $N\underline{H}_2$), 6.65 (1 H, d, *J*=8, $C^5\underline{H}$), 7.45 (1 H, s, $C^2\underline{H}$), 7.54 (1 H, d, *J*=8, $C^6\underline{H}$); ^{13}C-NMR δ 51.7 ($O\underline{C}H_3$, 55.4 ($O\underline{C}H_3$), 111.0, 113.0 ($\underline{C}^3$ and $\underline{C}^6$), 119.3 ($\underline{C}^2$), 124.0 ($\underline{C}^1$), 141.1 ($\underline{C}^4$), 146.0 ($\underline{C}^5$), 167.3 ($\underline{C}$=O); FAB-MS *m/z* 182 (MH^+), $C_9H_{11}NO_3$ requires 181.

Methyl 4-(acridin-9-ylamino)-3-methoxybenzoate 114

9-Chloroacridine **96** (0.396 g, 1.85 mmol) and methyl 4-amino-3-methoxybenzoate **112** (0.337 g, 1.85 mmol) were stirred in anhydrous MeOH (40 mL) until all the solid had dissolved. Methane sulfonic acid (0.2 mL) was added and the reaction mixture was stirred for 18 h. The mixture was neutralised (aq. 2 M NaOH) and was evaporated *in vacuo*. The residue was partitioned between CH_2Cl_2 and water and the aq. layer was washed with CH_2Cl_2 (2 x 40 mL). The combined organic layers were dried over sodium sulphate and evaporated *in vacuo*. The residue was purified by flash column chromatography eluting with 5 % ethyl acetate-hexane to yield an orange solid **114** (0.630 g, 95 %). ^{1}H-NMR ($CDCl_3$) δ 3.93 (3 H, s, OCH_3), 3.98 (3 H, s, OCH_3), 6.31(1H, d, *J*=8, $C^{5'}\underline{H}$), 7.23, (1H, bs, $C^{6'}\underline{H}$), 7.44 (2H, bs, $C^1\underline{H}$, $C\underline{H}^8$), 7.64 (1H, s, $C^{2'}\underline{H}$), 7.74 (2H, bs, $C^3\underline{H}$, $C^6\underline{H}$), 8.03 (2 H, d, *J*=9, $C^4\underline{H}$, $C^5\underline{H}$), 8.20 (2H, bs, $C^2\underline{H}$, $C^7\underline{H}$) : ^{13}C-NMR δ 51.8 ($O\underline{C}H_3$), 55.9 ($O\underline{C}H_3$), 110.9 ($\underline{C}^{2'}$), 112.4 ($\underline{C}^{5'}$), 121.1, 121.9, 123.6 ($\underline{C}^1$.

$\underline{C}^8$), 123.7 ($\underline{C}^4$, $\underline{C}^5$), 125.2, 130.3 ($\underline{C}^3$, $\underline{C}^6$), 146.41, 166.9 (C=O); FAB-MS *m/z* 359 (MH^+), $C_{22}H_{18}N_2O_3$ requires 358.

4-(Acridin-9-ylamino)-3-methoxybenzoic acid 116

Methyl 4-(acridin-9-ylamino)-3-methoxybenzoate **114** (0.63 g, 1.76 mmol) was stirred in dioxane (20 mL) and aq. KOH solution (2 M, 20 mL) for 6 h. The reaction mixture was neutralised (aq. 2 M HCl) and dioxane removed *in vacuo*. The remaining aq. suspension was filtered and the filter cake was purified by flash column chromatography eluting with 10 % ethyl acetate-hexane to yield the title compound **116** (0.570 g, 85 %), an orange solid. ^{1}H-NMR ($(CD_3)_2SO$) δ 4.03 (3 H, s, $OC\underline{H}_3$), 6.50 (1 H, d, *J*=8, $C\underline{H}^{5'}$), 7.18-7.33 (2 H, m), 7.44 (1 H, d, *J*=8, $C\underline{H}^{6'}$), 7.76-7.83 (7 H, m); ^{13}C-NMR δ 55. 9 ($OC\underline{H}_3$), 111.0, 121.6, 122.1, 124.7, 125.4, 131.2, 149.2, 165.4; FAB-MS *m/z* 345 (MH^+), $C_{21}H_{16}N_2O_3$ requires 344.

***N*^{1}-[4-(Acridin-9-ylamino)-3-methoxybenzoyl]-*N*^{2},*N*^{3},*N*^{4}-tri(benzyloxycarbonyl) spermine 118**

4-(Acridin-9-ylamino)-3-methoxybenzoic acid **116** (0.340 g, 0.98 mmol), *N*^{1},*N*^{2},*N*^{3}-tri(benzyloxycarbonyl)spermine **71** (0.597 g, 0.98 mmol), DCC (0.303 g, 1.47 mmol), HOBt (7 mg, 0.05 equiv.) and THF (15 mL) were used in general procedure A for amide bond formation. The reaction mixture was stirred for 48 h and was purified by flash column chromatography on silica gel eluting with 10 % MeOH-CH_2Cl_2 to yield the title compound **118** as an orange oil (0.345 g, 38 %). ^{1}H-NMR ($CDCl_3$) δ 1.38-1.80 (8 H, m, 4 x $CH_2C\underline{H}_2CH_2$), 3.00-3.41 (12 H, m 6 x $C\underline{H}_2N$), 4.05 (3 H, s, $OC\underline{H}_3$), 5.06 (6 H, s, 3 x $C\underline{H}_2O$), 6.42 (1 H, d, *J*=8, $C\underline{H}^{5'}$), 7.19-7.47 (18 H, m, 3 x Ph, $\underline{C}^1$H, $\underline{C}^8$H, $\underline{C}^{6'}$H), 7.60

(1 H, bs, C$\underline{H}^{2'}$), 7.75 (2 H, t, *J*=7, C$^{3}\underline{H}$, C$^{6}\underline{H}$), 8.07 (2 H, d, *J*=9, C$^{4}\underline{H}$, C$^{5}\underline{H}$), 8.17 (2 H, d, *J*=9, C$^{2}\underline{H}$, C$^{7}\underline{H}$); ^{13}C-NMR δ 24.7,25.7, 30.8, 32.0, 33.6, 35.5, 37.4, 43.5, 46.3, 53.7, 56.1 ($\underline{C}H_3$), 66.1 (OC$\underline{H}_2$), 67.1 (OC$\underline{H}_2$), 110.0 (C$^{2'}$), 113.1 (C$^{5'}$), 119.6, 121.0, 123.8 ($\underline{C}^{1}$, $\underline{C}^{6}$), 125.0, 127.6, (Ph), 128.0 (Ph), 128.3 (Ph), 130.7 ($\underline{C}^{3}$, $\underline{C}^{6}$), 136.5 (Ph), 146.7, 148.1, 156.5, ($\underline{C}$=O), 157.2 ($\underline{C}$=O), 166.8 (N$\underline{C}$=O; FAB-MS *m/z* 931 (MH^+), $C_{55}H_{58}N_6O_8$ requires 930.

N^1-[4-(Acridin-9-ylamino)-3-hydroxybenzoyl]spermine 119

N^1-[4-(Acridin-9-ylamino)-3-methoxybenzoyl]-N^2,N^3,N^4-tri(benzyloxycarbonyl) spermine **118** (0.15 g, 0.16 mmol) was dissolved in dry CH_2Cl_2 (2 mL) and TMSI (0.08 mL, 0.53 mmol) was added in a dropwise fashion over 5 min. The reaction mixture was stirred for 48 h and was then neutralised (aq. 2 M NaOH). The solution was evaporated *in vacuo* and the residue was purified by flash column chromatography on neutral alumina (grade 4) eluting with 20 % MeOH-CH_2Cl_2 to yield the title compound **119** as an orange hygroscopic foam (0.077 g, 91 %).^{1}H NMR (D_2O) δ 1.03-1.35 (10 H, m), 1.43-1.72 (6 H, m), 1.78 (2 H, m), 2.06 (2 H, m), 6.68 (2 H, t, *J*=7), 6.89 (1 H, t, *J*=7), 7.11 (m, 2 H), 7.38, (1 H, m), 7.83, (1 H, m), 8.05 (2 H, d, *J*=9), 8.18 (2 H, d, *J*=9); FAB-MS *m/z* 515 (MH^+), $C_{30}H_{38}N_6O_2$ requires 514.

N^1-[4-(Acridin-9-ylamino)-3-methoxybenzoyl]-N^4-(*t*-butoxycarbonyl) spermine 120

4-(Acridin-9-ylamino)-3-methoxybenzoic acid **116** (0.228 g, 0.66 mmol), N^1-(*t*-butoxycarbonyl)spermine **69** (0.20 g, 0.66 mmol), DCC (0.205 g, 0.99 mmol), HOBt (4 mg, 0.05 equiv.) and DMF (1 mL) were used in general procedure A for amide bond formation. The reaction mixture was stirred for 24 h and was purified by flash column

chromatography on silica gel eluting with CH_2Cl_2-MeOH-concentrated aq. NH_3 (100:10:1, 50:10:1then 10:5:1) to yield the title compound **120** as an orange foam (0.153 g, 37 %). ^{1}H-NMR ($CDCl_3$) δ 1.40 (9 H, s, 3 x C$\underline{H}_3$), 1.49 (4 H, m), 1.66 (2 H, m), 1.76 (2 H, m), 2.51-2.71 (8 H, m), 2.90-3.12 (4 H, m), 4.07 (3 H, s, OC$\underline{H}_3$), 6.38 (1 H, d, *J*=8, C$\underline{H}^{5'}$), 7.25 (2 H, bs), 7.64 (1 H, bs, C$\underline{H}^{2'}$), 7.75 (2 H, t, *J*=7, C$^3\underline{H}$, C$^6\underline{H}$), 8.11 (2 H, d, *J*=9, C$^4\underline{H}$, C$^5\underline{H}$), 8.15 (2 H, d, *J*=9, C$^2\underline{H}$, C$^7\underline{H}$); FAB-MS *m/z* 629 (MH^+), $C_{36}H_{48}N_6O_4$ requires 628.

N^1-[4-(Acridin-9-ylamino)-3-methoxybenzoyl]-spermine tri-trifluoroacetate salt 108

N^1-[4-(Acridin-9-ylamino)-3-methoxybenzoyl]-N^4-(*t*-butoxycarbonyl) spermine **120** (0.09 g, 0.14 mmol) was stirred in trifluoroacetic acid (2 mL) and CH_2Cl_2 (2 mL) at 0 °C for 45 min. The solvents were removed *in vacuo*, as an azeotrope with water and MeOH and the residue was lyophilised to yield the title compound as an orange hygroscopic foam (0.121 g, 99 %). ^{1}H NMR (D_2O) δ 1.04-1.34 (10 H, m), 1.37-1.68 (4 H, m), 1.80 (2 H, m), 2.10 (2 H, m), 4.05 (3 H, s, OC$\underline{H}_3$), 6.70 (2 H, t, *J*=7), 6.89 (1 H, t, *J*=7), 7.13 (m, 2 H), 7.34, (1 H, m), 7.86, (1 H. m), 8.10 (2 H, d, *J*=9), 8.19 (2 H, d, *J*=9); RP-HPLC 90 % MeCN:10 % aq. TFA (0.1 %), λ = 224 nm, 8 min; FAB-MS *m/z* 529 (MH^+), $C_{31}H_{40}N_6O_2$ requires 528.

Activation of Resin

p-Alkoxybenzylalcohol resin (5 g, 3.25 mmol) was suspended in anhydrous CH_2Cl_2 (10 mL) and *N*-methylmorpholine in CH_2Cl_2 was added (5 mL of a 1.3 M solution). 4-Nitrophenyl chloroformate (6.55g; 32.5 mmol) was dissolved in CH_2Cl_2 (30 mL) and

added to the reactor which was flushed with N_2 and was agitated for 16 h. The resin was repeatedly washed with anhydrous CH_2Cl_2 (10 x 40 mL), under N_2, until the washes showed no trace of reagent (spotted on TLC plate and visualised by UV light). The resin was suspended in CH_2Cl_2 (10 mL) and was agitated for a further 3 h with half quantities of reagents. The resin was washed, under N_2, with anhydrous CH_2Cl_2 (10 x 40 mL) until the washes showed no traces of reagent. The resin was dried *in vacuo* at 40 °C over P_2O_5. IR 3030, 2700, 1770.

Resin 127

The activated resin was suspended in anhydrous CH_2Cl_2 (25 mL) and a solution of N^1-*t*-butoxycarbonyl-N^1-(3-aminopropyl)-1,3-diaminopropane (5.0 g , 21.6 mmol) in anhydrous CH_2Cl_2 (10 mL) was added to the suspended resin then the reactor was sealed under N_2 and was agitated for 16 h. The resin was washed with CH_2Cl_2, THF, THF/H_2O (1:1) using 5 x 40 mL of each solvent and was then suspended in THF/H_2O (1:1) and was agitated for 48 h. The resin was washed with THF/H_2O (1:1) then THF (both 5 x 40 mL) then CH_2Cl_2 and then Et_2O (both 3 x 40 mL). The resin was dried *in vacuo* at 40 °C over P_2O_5. ^{13}C NMR (gel phase in $CDCl_3$): 28.40 (3 x CH), 31.6, 32.40 (2 x $CH_2\underline{C}H_2CH_2$), 39.4 ($\underline{C}NCO_2$), 40.5 ($\underline{C}NH_2$), 43.9 ($\underline{C}NBoc\underline{C}$), 79.8 ($\underline{C}(CH_3)_3$), 155.9 (C=O); IR 3030, 2900, 1940, 1870, 1800.

Acridine-9-acyl chloride hydrochloride 128

9-Acridinecarboxylic acid hydrate **73** (320 mg, 1.3 mmol) was suspended in toluene (2 mL). DMF (2 drops) and thionyl chloride (0.49 mL, mmol) were added to the suspension and the reaction mixture was heated to 80 °C for 45 min after which time all the reactants were in solution. The reaction mixture was evaporated *in vacuo* and the

yellow residue was washed with anhydrous Et_2O (70 mL). The filter cake was dried *in vacuo* at 40 °C over P_2O_5 and was used without further purification. IR 2230, 1950, 1780.

Acylation with 128

Resin **127** (400 mg) was swollen in DMF (4 mL) for 15 min. Acridine-9-acyl chloride (160 mg, 0.58 mmol) was dissolved in DMF (2 mL) and *N*-methylmorpholine in CH_2Cl_2 (0.9 mL of a 1.3 M solution) was added. This solution was added to the resin and the reactor was sealed, with an atmosphere of N_2, and was agitated for 16 h. The resin was washed with CH_2Cl_2 (5 x 5 mL) and was dried *in vacuo* at 40 °C over P_2O_5. The resin was reacted again with the same quantities of reagents for the same length of time and was washed and dried in the same way. (IR 2890, 1940, 1870, 1800). The product was cleaved from the resin using general procedure C. TLC of the residue showed only uncoupled polyamine.

Acylation with 128

Resin **127** (400 mg) was swollen in DMF (4 mL) for 15 min. Acridine-9-acyl chloride **128** (160 mg, 0.58 mmol) was dissolved in DMF (2 mL) and TEA (0.4 mL) was added. This solution was added to the resin and the reactor was sealed, with an atmosphere of N_2, and was agitated for 16 h. The resin was washed with CH_2Cl_2 (5 x 5 mL) and was dried *in vacuo* at 40 °C over P_2O_5. The resin was reacted again with the same quantities of reagents for the same length of time and was washed and dried in the same way. The product was cleaved from the resin using general procedure C. TLC of the residue showed only uncoupled polyamine.

Acylation with 2-mercaptothiazoline activated acridine-9-carboxlic acid 129

Acridine-9-acylchloride hydrochloride **128** (290 mg, 1.04 mmol) was suspended in anhydrous CH_2Cl_2 (6 mL) and TEA (0.3 mL, 2.08 mmol) was added. 2-Mercaptothiazoline (157 mg, 1.32 mmol) and DMAP (1 mg) were added and the solution was stirred for 1.5 h. Resin **127** (400 mg) was swollen with anhydrous CH_2Cl_2 (2 mL) and a portion of the solution of activated acridine (3 mL) was added. The reactor was sealed under N_2 and was agitated for 16 h. The resin was washed with anhydrous CH_2Cl_2 (5 x 5 mL) and was reacted with a further portion of the solution of activated acridine (3 mL) as before. The resin was washed with CH_2Cl_2, MeOH, CH_2Cl_2 (5 x 5 mL) of each solvent and was dried *in vacuo* at 40 °C over P_2O_5. (IR 3020,2890, 1940, 1870, 1800). The product was cleaved from the resin using general procedure C. TLC of the residue showed only uncoupled polyamine.

HOBt activated acridine-9-carboxylic acid 130

Acridine-9-carboxylic acid hydrate **73** (1.1 g, 5.6 mmol) was suspended in anhydrous DMF (20 mL) then DCC (1.43 g, 6.86 mmol) in anhydrous DMF (5 mL) and HOBt (680 mg, 5.03 mmol) in anhydrous DMF (5 mL) were added to the suspension. The reaction was stirred for 18 h at 60 °C and was then cooled and filtered. The filtrate was used without further treatment in acylation reactions.

Acylation with 130

Resin **127** (400 mg) was mixed with the solution of HOBt activated 9- acridine carboxylic acid **130** (0.6 mmol) in DMF (3.5 mL) then the reactor was flushed with N_2, sealed and agitated for 16 h. The resin was washed with anhydrous DMF (5 x 5 mL) and the procedure was repeated with the same quantity of reagent, agitating for 3 h. The

resin **131** was washed successively with DMF, THF, THF/H_2O (1:1), THF then Et_2O (5 x 5 mL each solvent) and was then dried *in vacuo* at 40°C over P_2O_5. (IR 3310, 3020, 1940, 1870, 1800). The product was cleaved from the resin **131** using general procedure D. The residue was purified by flash column chromatography on silica gel eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (8:5:1) to yield the product **132** as a pale yellow gum (80 mg). ^{1}H-NMR (D_2O) δ 2.18 (2 H, m, $CH_2C\underline{H}_2CH_2$), 2.27 (2 H, m, $CH_2C\underline{H}_2CH_2$), 3.15 (2 H, m, $C\underline{H}_2N$), 3.27 (2 H, m, $C\underline{H}_2N$), 3.31 (2H, m, $C\underline{H}_2N$), 3.80 (2 H, m, $C\underline{H}_2NHC{=}O$), 7.82 (2 H, t, *J*=7,), 8.09 (2 H, t, *J*=7), 8.14 (2 H, d, *J*=8), 8.26 (2 H, d, *J*=8); FAB-MS *m/z* 337 (MH^+), $C_{20}H_{24}N_4O$ requires 336; TLC R_f 0.60 (4:2:1, CH_2Cl_2/MeOH/NH_4OH); IR 3250, 1640, 1470.

Resin 137

The resin **127** (2.0 g) was swollen in anhydrous DMF/EtOH (3:1) (12 mL) and 4-azidobutanal **134** (1.2 g, 10.6 mmol) was dissolved in anhydrous DMF (2 mL) and was added to the suspended resin. BAP (8 M in CH_2Cl_2, 1.3 mL) was added to the reaction mixture. The reactor was flushed with Ar before being sealed and agitated for 48 h. The resin **137** was washed with DMF, THF, THF/H_2O (1:1), THF, Et_2O (5 x 5 mL each solvent). The resin was dried *in vacuo* at 40 °C over P_2O_5. IR 3330, 2030, 2090, 1940, 1880, 1800.

Resin 136

The resin **127** (2.0 g) was swollen in anhydrous DMF/EtOH (3:1) (12 mL) and 5-azidopentan-2-one **133** (960 mg, 7.6 mmol) was dissolved in anhydrous DMF (2 mL) and was added to the suspended resin. BAP (8 M BH_3, 1.3 mL) was added to the reaction mixture then the reactor was flushed with Ar before being sealed and agitated

for 48 h. The resin was washed with DMF, THF, THF/H_2O (1:1), THF, Et_2O (5 x 5 mL each solvent). The resin **136** was dried *in vacuo* at 40 °C over P_2O_5. IR 3030, 2090, 1940, 1880, 1800.

Resin 138

The resin **127** (2.0 g) was swollen in anhydrous DMF/EtOH (3:1) (12 mL) and the 3-(Pnt-amino)propanal **135** (1.2 g, 7.73 mmol) was dissolved in anhydrous DMF (2 mL) and was added to the suspended resin. BAP (8 M in CH_2Cl_2, 1.3 mL) was added to the reaction mixture then the reactor was flushed with Ar before being sealed and agitated for 48 h. The resin **138** was washed with DMF, THF, THF/H_2O (1:1), THF, Et_2O (5 x 5 mL each solvent). The resin was dried *in vacuo* at 40 °C over P_2O_5. IR 3330, 3030, 1940, 1870, 1800.

Resin 139

Resin **136** (1.0 g) was swollen in CH_2Cl_2 (5 mL) and Boc anhydride (725 mg, 3.32 mmol) was added. The reactor was flushed with N_2 and sealed and was then agitated for 15 h. The resin was washed with CH_2Cl_2 (5 x 5 mL) and was reacted a second time with the same quantity of Boc anhydride. The resin **139** was washed with CH_2Cl_2, THF, THF/H_2O (1:1) and THF (5 x 5 mL each solvent) and was dried *in vacuo* at 40 °C over P_2O_5. IR 3330, 3030, 2090, 1940, 1880, 1800.

Resin 142

Resin **139** (1 g) was used in the general procedure E for azide reduction. IR 3360, 2950, 1940, 1870, 1800.

Resin 145

Resin **142** was used in the general procedure F for HOBt activated 9-acridinecarboxylic acid acylation. IR 3310, 3020, 1940, 1870, 1800.

***N*-(3-(4-(Acridine-9-ylcarbonylamino)-1-methylbutylamino)propyl)-1,3-diamine 123**

Resin **145** was used in general procedure D for resin cleavage. The residue was purified by flash column chromatography on silica gel eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (8:5:1) to yield the product **123** as a pale yellow gum (9 mg). ^{1}H-NMR (D_2O) δ 1.42 (3 H, s, C<u>H</u>$_3$), 2.15-2.21 (8 H, m, 4 x $CH_2C\underline{H}_2CH_2$), 3.04-3.32 (8 H, 4 x C<u>H</u>$_2$N), 3.42-3.53 (1 H, m, C<u>H</u>CH_3), 3.79 (2 H, t, C<u>H</u>$_2$NHC=O), 7.98 (2 H, t, *J*=7), 8.31 (4 H, m), 8.48 (2 H, d, *J*=8); FAB-MS *m/z* 422 (MH^+), $C_{25}H_{35}N_5O$ requires 421.

Resin 148

Resin **136** (1.0 g) was used in general procedure F for acridine acylation. IR 3330, 2850, 2090, 1950, 1880, 1800.

Resin 151

Resin **148** was used in general procedure E for azido reduction. IR 3340, 3030, 1940, 1870, 1800.

Cleavage of Resin 151

Resin **151** was used in general procedure D for resin cleavage. TLC of the crude product showed only uncoupled polyamines.

Resin 140

Resin **137** (1.0 g) was swollen in CH_2Cl_2 (7 mL) and Boc anhydride (725 mg, 3.32 mmol) was added. The reactor was flushed with N_2 and sealed and was then agitated for 15 h. The resin was washed with CH_2Cl_2 (5 x 5 mL) and was reacted a second time with the same quantity of Boc anhydride. The resin **140** was washed with CH_2Cl_2, THF, THF/H_2O (1:1) and THF (5 x 5 mL each solvent) and was dried *in vacuo* at 40 °C over P_2O_5. IR 3330, 3030, 2090, 1940, 1880, 1800.

Resin 143

Resin **140** was used in general procedure E for azide reduction. IR 3360, 2950, 1940, 1870, 1800.

Resin 146

Resin **143** was used in general procedure F for HOBt activated acridine-9-carboxylic acid acylation. IR 3310, 3020, 1940, 1870, 1800.

N^1-(4-(Acridine-9-ylcarbonylamino)butyl)-N^2-(3-aminopropyl)propan-1,3-diamine 125

Resin **146** was used in the general procedure for resin cleavage. The residue was purified by flash column chromatography on silica gel eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (8:5:1) to yield the product as a pale yellow gum. ^{1}H-NMR (D_2O) δ 2.08-2.20 (8 H, m, 4 x $CH_2C\underline{H}_2CH_2$), 3.05-3.30 (8 H, 4 x $C\underline{H}_2N$), 3.42-3.53 (2 H, m, $C\underline{H}_2NH_2$), 3.60 (2 H, m, $C\underline{H}_2NHC{=}O$), 7.87 (2 H, t, *J*=7), 8.27 (4 H, m), 8.46 (2 H, d, *J*=8); FAB-MS *m/z* 408 (MH^+), $C_{24}H_{33}N_5O$ requires 407; IR 3260, 1640, 1480.

Resin 149

Resin **137** (1.0 g) was used in general procedure F for acridine acylation. IR 3330, 2850, 2090, 1950, 1880, 1800.

Resin 152

Resin **149** was used in general procedure E for azido reduction. IR 3340, 3030, 1940, 1870, 1800.

Cleavage of Resin 152

Resin **152** was used in general procedure D for resin cleavage. TLC of the crude product showed only uncoupled polyamines.

Resin 141

Resin **138** was swollen in CH_2Cl_2 (5 mL) and Boc anhydride (725 mg,) was added. The reactor was flushed with N_2 and sealed and was then agitated for 15 h. The resin was washed with CH_2Cl_2 (5 x 5 mL) and was reacted a second time with the same quantity of Boc anhydride. The resin **141** was washed with CH_2Cl_2, THF, THF/H_2O (1:1) and THF (5 x 5 mL) and was dried *in vacuo* at 40 °C over P_2O_5. IR 3330, 3030, 1940, 1880, 1800.

Resin 144

Iodine (3.3g, 13mmol) was dissolved in THF/$H_2$0 (1:1) (7 mL) and the resin **141** was swollen in the solution. The reactor was sealed and was agitated for 18 h then the resin was successively washed with THF, THF/H_2O (1:1), THF/sat. aq. $Na_2S_2O_3$, THF,

CH_2Cl_2 then Et_2O (5 x 5 mL each solvent). The resin **144** was dried *in vacuo*, over P_2O_5. IR 3330, 3030, 1940, 1880, 1800.

Resin 147

Resin **144** was used in general procedure F for HOBt activated 9-acridinecarboxylic acid acylation. IR 3310, 3020, 1940, 1870, 1800.

N^1-(3 -(Acridine-9-ylcarbonylamino)propyl)-N^2-(3-aminopropyl)propan-1,3-diamine 121

Resin **146** was used in the general procedure for resin cleavage. The residue was purified by flash column chromatography on silica gel eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (8:5:1) to yield the product **121** as a pale yellow gum. ^{1}H-NMR (D_2O) δ 2.10-2.18 (6 H, m, 3 x $CH_2C\underline{H}_2CH_2$), 3.05-3.25 (8 H, 4 x $C\underline{H}_2N$), 3.37-3.45 (2 H, m, $C\underline{H}NH_2$), 3.55 (2 H, m, $C\underline{H}_2NHC{=}O$), 7.82 (2 H, t, *J*=7), 8.35 (4 H, m), 8.48 (2 H, d, *J*=8); FAB-MS *m/z* 394 (MH^+), $C_{23}H_{31}N_5O$ requires 393; IR 3260, 1640, 1480.

Resin 150

Resin **138** (1.0 g) was used in general procedure F for acridine acylation. IR 3330, 3030, 1940, 1880, 1800.

Resin 153

Iodine (3.3 g, 13 mmol) was dissolved in THF/H_20 (1:1) (7 mL) and the resin **150** was swollen in the solution. The reactor was sealed and was agitated for 18 h then the resin was successively washed with THF, THF/H_2O (1:1), THF/sat. aq. $Na_2S_2O_3$, THF,

CH_2Cl_2 then Et_2O (5 x 5 mL each solvent). The resin **153** was dried *in vacuo*, over P_2O_5. IR 3330, 3030, 1940, 1880, 1800.

Cleavage of Resin 153

Resin **153** was used in general procedure D for resin cleavage. TLC of the crude product showed only uncoupled polyamines.

Isoferulic acid 173 (Blase and Banerjee, 1995)

3-Hydroxy-4-methoxybenzaldehyde **172** (5.00 g, 32.9 mmol) and malonic acid (7.75 g, 74.4 mmol) were dissolved in piperidine (0.5 mL) and pyridine (30 mL). The solution was heated to 80 °C for 1 h and was allowed to cool before being poured into water (200 mL). This solution was acidified with 2 N aq. HCl and the precipitate was collected by filtration. This product (6.02 g, 94 %) was dried *in vacuo* over P_2O_5 (MP 236-238 °C; lit. 238-240 °C Stoll and Seebeck, 1957). ^{1}H-NMR (D_6-DMSO) δ 3.77 (3H, s, $OC\underline{H}_3$), 6.24 (1H, d, *J*=16, $C^{2'}\underline{H}$), 6.92 (1H, d, *J*=8, $C^6\underline{H}$), 7.5-7.10 (2 H, m, $C^5\underline{H}$, $C^2\underline{H}$), 7.45 (1H, d, *J*=16, $C^{1'}\underline{H}$); ^{13}C-NMR δ 55.7 ($O\underline{C}H_3$), 111.9 ($\underline{C}^5$), 114.0 ($\underline{C}^2$), 116.3 ($\underline{C}^6$), 121.1 ($\underline{C}^{2'}$), 127.1 ($\underline{C}^1$), 144.0 ($\underline{C}^{1'}$), 146.8 (C^3), 149.5 ($\underline{C}^4$), 177.1 ($\underline{C}$=O); EI-MS *m/z* 194 (M^+), $C_{10}H_{10}O_4$ requires 194; Found C 61.70, H 5.20, N 0.00, requires C 61.85, H 5.19, N 0.00 %.

4-Fluorocinnamic acid 177 (Luo *et al.*, 1990)

4-Fluorobenzaldehyde **174** (17.3 mL, 160 mmol) and malonic acid (21.4 g, 480 mmol) were dissolved in a mixture of piperidine (3 mL) and pyridine (200 mL). The solution was heated to 80 °C for 4.5 h and was then cooled to room temperature. The solution was poured onto crushed ice (500 g) and the mixture was acidified with conc. aq. HCl.

Once the ice melted the mixture was filtered and the filtrate, a purple solid, was recrystallised from EtOH to yield white needles MP BERGGMAN(23.6 g, 89 %). ^{1}H-NMR (d_6 DMSO) δ 6.54 (1H, d, *J*=16, $C^{2'}\underline{H}$), 7.27 (2H, t, *J*=9, $C^2\underline{H}$, $C^6\underline{H}$), 7.64 (1H, d, *J*=16, $C^{1'}\underline{H}$), 7.79 (2H, dd, J_{HH}=9, J_{HF}=11, $C^3\underline{H}$, $C^5\underline{H}$); ^{13}C-NMR δ 116.2 (d, *J*=22, $\underline{C}^3$, $\underline{C}^5$), 119.3 ($\underline{C}^{2'}$), 130.7 (d, *J*=8, $\underline{C}^2$, $\underline{C}^6$), 131.1 (d, *J*=2, $\underline{C}^1$), 143.0 ($\underline{C}^{1'}$), 163.4 (d, *J*=220, $\underline{C}^4$), 167.9 ($\underline{C}$=O); FAB-MS *m/z* 167 (MH^+), $C_9H_7FO_2$ requires 166; Found C 65.00, H 4.34, N 0.00, requires C 65.06, H 4.25, N 0.00 %.

4-Fluoro-3-nitrocinnamic acid 176

4-Fluorocinnamic acid **177** (3.7 g, 22.3 mmol) was added to conc. HNO_3 over 5 min and the mixture was stirred for a further 1 h. The reaction mixture was the poured over crushed ice (200 g) and the precipitate formed was collected by filtration once the ice had melted. The filtrate was washed well with water and was dried *in vacuo* over P_2O_5. The product was a white solid (4.0 g, 85 %). ^{1}H-NMR (d_6 DMSO) δ 6.53 (1 H, d, *J*=16, $C^{2'}\underline{H}$), 7.68 (1 H, td, *J*=8, *J*=2, $C^5\underline{H}$), 7.79 (1H, d, *J*=16, $C^{1'}\underline{H}$), 7.97-8.08 (2 H, m, $C^2\underline{H}$, $C^6\underline{H}$); ^{13}C-NMR δ 112.6 (d, *J*=27, C^3), 121.5 (d, *J*=22, $\underline{C}^5$), 124.2 ($\underline{C}^{2'}$), 126.2 (d, *J*=4, $\underline{C}^1$), 131.7 (d, *J*=9, $\underline{C}^{2/6}$), 138.1 ($\underline{C}^{1'}$), 149.1 (d, *J*=9, $\underline{C}^{2/6}$), 162.1 (d, *J*=252, $\underline{C}^4$), 167.1 ($\underline{C}$=O); EI-MS *m/z* 211 (M^+), $C_9H_6FNO_4$ requires 211; Found: C 49.80, H 2.42, N 8.31 requires C 49.72, H 2.38, N 8.28 %.

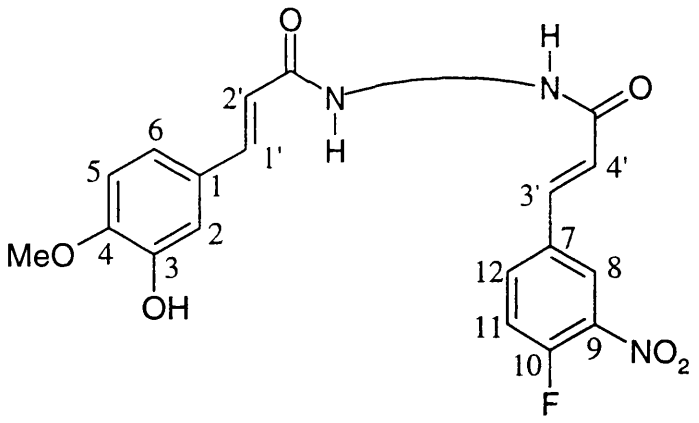
***N*^{1}-*t*-Butoxycarbonyl-*N*^{1}-(3-aminopropyl)-1,3-diaminopropane 181**

N-(3-Aminopropyl)-1,3-diamine **178** (5.00 g, 38.2 mmol) was dissolved in THF (15 mL) then ethyl trifluoroacetate (9.1 mL, 76.3 mmol) was added over 5 min. The reaction mixture was stirred for 1 h then Boc anhydride (12.5 g, 57.3 mmol) was added. The reaction mixture was stirred for a further 18 h then conc. aq. NH_3 was added to the

reaction mixture until the pH was >11. The reaction mixture was stirred for 24 h until TLC showed the reaction was complete. The reaction mixture was evaporated *in vacuo* and the residue was purified by flash column chromatography on silica gel eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (15:10:1) to yield the title compound **181** as a colourless oil (5.47 g, 62 %). ^{1}H-NMR ($CDCl_3$) δ 1.47 (9H, s, C($C\underline{H}_3$)$_3$), 1.66 (4H, quin., *J*=6, 2 x $CH_2C\underline{H}_2CH_2$), 2.68 (8H, t, *J*=7, 2 x $C\underline{H}_2N\underline{H}_2$), 3.27 (4 H, bs, $C\underline{H}_2NBocC\underline{H}_2$); ^{13}C-NMR δ 28.5 (3 x $\underline{C}H_3$), 30.8 ($CH_2\underline{C}H_2CH_2$), 31.8 ($CH_2\underline{C}H_2CH_2$), 38.5 ($\underline{C}H_2NH_2$), 39.1 ($\underline{C}H_2NH_2$), 43.6 ($\underline{C}H_2NBoc$), 44.3 ($\underline{C}H_2NBoc$), 79.3 ($\underline{C}(CH_3)_3$), 155.6 ($\underline{C}$=O); FAB-MS *m/z* 232 (MH^+) $C_{11}H_{25}N_3O_2$ requires 231.

N^1-*t*-Butoxycarbonyl-N^1-(3-(3-hydroxy-4-methoxycinnamoylamino)propyl)-1,3-diaminopropane 182

3-Hydroxy-4-methoxycinnamic acid **173** (140 mg, 0.72 mmol) was dissolved in THF (5 mL). DCC (164 mg, 0.80 mmol), 2-mercaptothiazolidine (95 mg, 0.80 mmol) and DMAP (1 mg) were added and the reaction mixture was stirred for 1 h. The white precipitate was removed from the yellow solution by filtration. N^1-*t*-Butoxycarbonyl-N^1-(3-aminopropyl)-1,3-diaminopropane **181** (333 mg, 1.44 mmol) was dissolved in THF (30 mL) and the yellow filtrate was added in one portion. The reaction mixture was stirred for 10 h and was then evaporated *in vacuo*. The residue was purified by flash column chromatography on silica gel eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (50:10:1) to yield the title compound **182** as a white wax (61 mg, 75 %). ^{1}H-NMR ($CDCl_3$) δ 1.39 (9 H, s, C($C\underline{H}_3$)$_3$), 1.56-1.72 (4 H, m, $CH_2C\underline{H}_2CH_2$), 2.88-3.03 (4 H, m, $C\underline{H}_2NBocC\underline{H}_2$), 3.10-3.33 (4 H, m, $C\underline{H}_2NH_2$, $C\underline{H}_2NHC$=O), 3.81 (3 H, s, $OC\underline{H}_3$), 6.26 (1 H, d, *J*=16, $C^{2'}\underline{H}$), 6.73 (1 H, d, *J*=8, $C^6\underline{H}$), 6.83-6.94 (1 H, m, $C^5\underline{H}$), 7.06 (1H, s, $C^2\underline{H}$), 7.42 (1 H, d, *J*=16, $C^{1'}\underline{H}$); FAB-MS *m/z* 167 (MH^+), $C_9H_7FO_2$ requires 166.



N^1-*t*-Butoxycarbonyl-N^1-(3-(3-hydroxy-4-methoxycinnamoylamino)propyl)-N^2-(4-fluoro-3-nitrocinnamoyl)-1,3-diaminopropane 183

4-Fluoro-3-nitrocinnamic acid **176** (97 mg, 0.46 mmol) was dissolved in THF (5 mL). DCC (113 mg, 0.65 mmol), 2-mercaptothiazolidine (60 mg, 0.50 mmol) and DMAP (1 mg) were added and the reaction mixture was stirred for 1 h. The white precipitate was removed from the yellow solution by filtration. N^1-*t*-butoxycarbonyl-N^1-(3-(3-hydroxy-4-methoxycinnamoylamino)propyl)-1,3-diaminopropane **182** (160 mg, 0.46 mmol) was dissolved in THF (5 mL) and was added to the yellow filtrate. The reaction mixture was stirred for 10 h and was then evaporated *in vacuo*. The residue was purified by flash column chromatography on silica gel eluting with 5 % MeOH/CH_2Cl_2 to yield the title compound **183** as a white wax (190 mg, 69 %). ^{1}H-NMR ($CDCl_3$) δ 1.41 (9H, s, 3 x $C\underline{H}_3$), 1.61-1.85 (4 H, m, 2 x $CH_2C\underline{H}_2CH_2$), 3.08-3.43 (8 H, 4 x $C\underline{H}_2N$), 3.85 (3 H, s, $OC\underline{H}_3$), 6.29 (1 H, d, J=16, $C^{2'}\underline{H}$), 6.38 (1 H, d, J=16, $C^{4'}\underline{H}$), 6.79 (1 H, d, J=8, $C^3\underline{H}$), 6.93 (1 H, dd, J=8, J=1, $C^{12}\underline{H}$), 7.08 (1 H, d, j=2, $C^8\underline{H}$), 7.27 (1 H, s, $C^6\underline{H}$), 7.48 (1 H, d, *J*=16, $C^{3'}\underline{H}$), 7.57 (1 H, bs, $C^2\underline{H}$), 7.71 (1 H, dd, J=8, J=2, $C^{11}\underline{H}$), 7.95 (1H, d, j=16, $C^{1'}\underline{H}$); ^{13}C-NMR δ; 28.3 ($\underline{C}H_3$), 36.3 (b), 37.1 (b), 44.7 (b), 55.8 ($\underline{C}(CH_3)_3$), 80.0 ($O\underline{C}H_3$), 110.8 ($\underline{C}^5$), 112.9 ($\underline{C}^2$), 118.8 ($\underline{C}^6$), 120.8 (d, *J*=20, $\underline{C}^{11}$), 121.3 ($\underline{C}^{2'}$), 126.4 ($\underline{C}^{4'}$), 127.3 (d, *J*=3, $\underline{C}^7$), 128.0 ($\underline{C}^1$), 130.7 (d, *J*=9, $\underline{C}^{12}$), 132.6 ($\underline{C}^9$), 134.7 ($\underline{C}^{3'}$), 140.5

($\underline{C}^{1'}$), 146.0 ($\underline{C}^{3}$), 148.5 ($\underline{C}^{4}$), 158.2 (d, *J*=258, $\underline{C}^{10}$), 163.8 (<u>C</u>=O), 165.0 (<u>C</u>=O), 166.7 (<u>C</u>=O); FAB-MS *m/z* 601 (MH^+), $C_{30}H_{37}FN_4O_8$ requires 600; FAB-HRMS calcd. 601.2474 (MH^+), found 601.2686.

_N_-Boc-_O_-methyl-2'-nitronorcadabicine 170

N^1-*t*-Butoxycarbonyl-N^1-(3-(3-hydroxy-4-methoxycinnamoylamino)propyl)-N^2-(4-fluoro-3-nitrocinnamoyl)-1,3-diaminopropane **183** (90 mg, 0.16 mmol) and CsF (121 mg, 0.80 mmol) were combined in anhydrous DMF (16 mL) and were stirred for 18 h. H_2O (30 mL) was added to the reaction mixture and this solution was washed with EtOAc (3 x 30 mL). The combined organic washes were dried ($MgSO_4$) and evaporated *in vacuo*. The residue was purified by RP-HPLC (1:4 aq. TFA (0.1 %)/MeOH, λ=250 nm) to yield the product **170** as a buff foam (73 mg, 79 %). ^{1}H-NMR ($CDCl_3$) δ 1.48 (9 H, s, $C(C\underline{H}_3)_3$), 1.67-1.90 (4 H, m, $CH_2C\underline{H}_2CH_2$), 3.12-3.47 (8 H, m, $C\underline{H}_2N$), 3.90 (3 H, s, $OC\underline{H}_3$), 6.31 (1H, d, *J*=16), 6.42 (1H, d, *J*=16), 6.78 (1H, d, *J*=8), 6.92 (1H, d, *J*=8), 7.09 (1 H, s), 7.48 (1 H, d, *J*=16), 7.53-7.64 (2 H, m), 7.71 (1H, d, *J*=8), 7.94 (1H, d, *J*=16); ^{13}C-NMR δ; 28.4 ($\underline{C}H_3$), 36.2, 37.0, 44.6, 55.9 ($\underline{C}(CH_3)_3$), 89.8 ($O\underline{C}H_3$), 110.7, 112.1, 112.6, 118.8, 120.6, 120.9, 121.5, 126.7, 127.6, 128.2, 131.3, 132.9 , 135.0, 140.6, 145.9, 148.5, 160.1 (<u>C</u>=O), 164.3 (<u>C</u>=O), 165.0 (<u>C</u>=O); FAB-MS *m/z* 581 (MH^+), $C_{30}H_3N_4O_8$ requires 580.

4-(Tetrahydropyrimidin-1-yl)butylamine 184 (Almedia *et al.*, 1988)

Spermidine **2** (2.0 g, 13.8 mmol) was dissolved in H_2O (50 mL) and the solution was cooled to 5 °C. 36 % aq. formaldehyde solution (1.0 mL, 12.4 mmol) was added over 5 min then the reaction mixture was allowed to warm to 20°C and was stirred for 1 h. NaCl was added until the solution was saturated and it was then extracted with $CHCl_3$

(4 x 40 mL). The combined organic extracts were dried ($MgSO_4$) and evaporated *in vacuo* to give a colourless viscous oil (1.93 g, 89 %) which was used without further purification. ^{1}H-NMR ($CDCl_3$) δ 1.41-1.71 (6H, m, 3 x $CH_2\underline{CH_2}CH_2$), 2.18-2.30 (2H, m, $C\underline{H_2}N$), 2.46-2.63 (2H, m, $C\underline{H_2}N$), 2.68-2.85 (4H, $NC\underline{H_2}CH_2C\underline{H_2}N$), 3.30-3.50 (5H, $NC\underline{H_2}N$, 3 x $N\underline{H}$); ^{13}C-NMR δ 24.0 ($CH_2\underline{C}H_2CH_2CH_2$), 26.6 ($CH_2\underline{C}H_2CH_2CH_2$), 30.4 ($CH_2\underline{C}H_2CH_2$), 41.1 ($\underline{C}H_2N$), 44.5 ($\underline{C}H_2N$), 52.7 ($N\underline{C}H_2CH_2CH_2N$), 55.0 ($N\underline{C}H_2CH_2CH_2N$), 69.5 ($N\underline{C}H_2N$); FAB-MS *m/z* 158 ($MH^+$) $C_8H_{19}N_3$ requires 157.

***N*-(3-Hydroxy-4-methoxycinnamoyl)-4-(tetrahydropyrimidin-1-yl)butylamine 185**

3-Hydroxy-4-methoxycinnamic acid **176** (435 mg, 2.24 mmol) was suspended in a mixture of $CHCl_3$ (10 mL) and CH_2Cl_2 (10 mL). DCC (690 mg, 3.36 mmol), 2-mercaptothiazolidine (294 mg, 2.46 mmol) and DMAP (1 mg) were added and the reaction mixture was stirred for 1 h. The white precipitate was removed from the yellow solution by filtration. 4-(Tetrahydropyrimidin-1-yl)butylamine **184** (700 mg, 4.48 mmol) was dissolved in a mixture of $CHCl_3$ (20 mL) and CH_2Cl_2 (20 mL) and this solution was cooled to -78 °C. The yellow filtrate was added and the solution was allowed to warm to 20 °C and was stirred for 5 h. The reaction mixture was evaporated *in vacuo* and the residue was purified by flash column chromatography on silica gel eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (200:10:1, 100:10:1 then 50:10:1) to yield the title compound **185** as a white foam (410 mg, 55 %). ^{1}H-NMR ($CDCl_3$) δ 137-1.73 (6H, m, 3 x $CH_2\underline{CH_2}CH_2$), 2.08-2.30 (2H, m, $C\underline{H_2}N$), 2.50 (2H, bs, $C\underline{H_2}N$), 2.74 (2H, bs, $C\underline{H_2}N$), 3.08-3.42 (4 H, m, $NC\underline{H_2}N$, $C\underline{H_2}NHC{=}O$), 3.79 (3 H, s, $OC\underline{H_3}$), 6.36 (1 H, d, *J*=16,), 6.71 (1 H, d, *J*=8,), 6.86 (1 H, d, *J*=8), 7.08 (1 H, s,), 7.46 (1 H, d, *J*=16); ^{13}C-NMR δ:23.7 ($CH_2\underline{C}H_2CH_2CH_2$), 25.2 ($CH_2\underline{C}H_2CH_2CH_2$), 27.2 ($CH_2\underline{C}H_2CH_2$), 39.2, 44.5 ($\underline{C}H_2N$), 52.5 ($\underline{C}H_2N$), 54.6 ($CH_2NHC{=}O$), 55.6 ($O\underline{C}H_3$), 68.7 ($N\underline{C}H_2N$), 111.0

($\underline{C}^5$), 113.3 ($\underline{C}^2$), 118.3 ($\underline{C}^6$), 121.1 ($\underline{C}^{2'}$), 127.6 ($\underline{C}^1$), 140.3 ($\underline{C}^{1'}$), 146.8 ($\underline{C}^3$), 149.4 ($\underline{C}^4$), 166.8 ($\underline{C}$=O); FAB-MS *m/z* 334 (MH^+) $C_{18}H_{27}N_3O_3$ requires 333.

N^1-3-Hydroxy-4-methoxycinnamoylspermidine 186

N-(3-Hydroxy-4-methoxycinnamoyl)-4-(tetrahydropyrimidin-1-yl)butylamine **185** (200 mg, 0.60 mmol), pyridine and malonic acid (225 mg, 2.16 mmol) were dissolved in anhydrous MeOH (5 mL). The reaction mixture was heated to reflux for 2 h and was then evaporated *in vacuo*. The residue was purified by flash column chromatography on silica gel eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (8:5:1) to yield the title compound **186** as a colourless glass (152 mg, 79 %). ^{1}H-NMR (CD_3OD) δ 1.21-1.57 (6H, m, 3 x $CH_2\underline{CH_2}CH_2$), 2.37-2.92 (6H, m, 2 x $\underline{CH_2}NH$, $\underline{CH_2}N$), 3.05 (2 H, m, $\underline{CH_2}NC$=O), 3.60 (3 H, s, $OC\underline{H_3}$), 6.17 (1 H, d, *J*=16, $\underline{C}^{2'}$), 6.61 (1 H, d, *J*=8,), 6.68 (1 H, d, *J*=8), 6.78 (1 H, s, $\underline{C}^2$), 7.14 (1 H, d, *J*=16, $\underline{C}^{1'}$); ^{13}C-NMR δ; 26.3, 27.5, 28.6, 29.1, 30.1, 32.3, 40.2, 41.1, 56.9 ($O\underline{C}H_3$), 112.8 ($\underline{C}^5$), 115.1 ($\underline{C}^2$), 119.3 ($\underline{C}^6$), 122.0 ($\underline{C}^{2'}$), 125.7 ($\underline{C}^1$), 142.2 ($\underline{C}^{1'}$), 146.8 ($\underline{C}^3$), 149.3 ($\underline{C}^4$), 169.2 ($\underline{C}$=O); FAB-MS *m/z* 322 (MH^+), $C_{17}H_{27}N_3O_3$ requires 321.

N^1-3-Hydroxy-4-methoxycinnamoyl-N^3-4-fluoro-3-nitrocinnamoylspermidine

4-Fluoro-3-nitrocinnamic acid **176** (73 mg, 0.34 mmol) was suspended in CH_2Cl_2 (10 mL) and DCC (106 mg, 0.51 mmol), 2-mercaptothiazolidine (45 mg, 0.38 mol) and DMAP (1 mg) were added. The reaction mixture was stirred for 1 h and was then filtered to remove the white precipitate from the yellow solution. N^1-3-Hydroxy-4-methoxycinnamoylspermidine **186** (110 mg, 0.34 mmol) was dissolved in MeOH (20 mL) and the solution was cooled to -78 °C. The yellow filtrate was added to this solution and the reaction mixture was allowed to warm to 20 °C and then stirred at this

temperature for 3 h. The reaction mixture was evaporated *in vacuo* and the residue was purified by flash column chromatography on silica gel eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (50:10:1) to yield the title compound as a pale brown glass (107 mg, 61 %). ^{1}H-NMR (CD_3OD) δ 1.48 (4H, bs, 2 x $CH_2C\underline{H}_2CH_2$), 1.63-1.81 (2H, m, $CH_2C\underline{H}_2CH_2$), 2.57-2.73 (4H, m, 2 x $C\underline{H}_2NH$), 3.23 (4 H, t, *J*=9, 2 x $C\underline{H}_2NC{=}O$), 374 (3 H, s, $OC\underline{H}_3$), 6.24 (1 H, d, *J*=16), 6.38 (1 H, d, *J*=16), 6.70-6.89 (3 H, m), 7.23 (1 H, d, *J*=16), 7.32 (1 H, td, *J*=8, *J*=2), 7.57 (2 H, m), 7.70 (1 H, d, *J*=16); FAB-MS *m/z* 515 (MH^+), $C_{26}H_{31}FN_4O_6$ requires 514.

N^2-t-Butoxycarbonyl-N^3-4-fluoro-3-nitrocinnamoyl-N^1-3-hydroxy-4-methoxycinnamoylspermidine 187

N^3-4-Fluoro-3-nitrocinnamoyl-N^1-3-hydroxy-4-methoxycinnamoylspermidine (100 mg, 0.19 mmol) and Boc anhydride (85 mg, 0.39 mmol) were mixed in MeOH (5 mL). The reaction mixture was stirred for 18 h and then water (5 mL) was added. After a further 4 h the reaction mixture was evaporated *in vacuo* and the residue was purified by flash column chromatography on silica gel eluting with 5 % CH_2Cl_2/MeOH to yield the title compound **187** as a pale brown glass (96 mg, 82 %). ^{1}H-NMR ($CDCl_3$) δ 1.43 (9 H, $C(C\underline{H}_3)_3$), 1.43-1.56 (6H, m, 3 x $CH_2C\underline{H}_2CH_2$), 1.56-1.96 (8H, m, 4 x $C\underline{H}_2N$), 3.89 (3 H, s, $OC\underline{H}_3$), 6.32 (1 H, d, *J*=16), 6.42 (1 H, d, *J*=16), 6.92 (1 H, dd, *J*=8, *J*=2), 7.08 (1 H, d, *J*=2), 7.24 (1 H, d, *J*=8), 7.48 (1H, d, *J*=16), 7.48-7.63 (2H, m), 7.70 (1H, dd, *J*=8, *J*=3), 7.94 (1H, d, *J*=16); δ; FAB-MS *m/z* 615 (MH^+), $C_{31}H_{39}FN_4O_8$ requires 614; FAB-HRMS calcd. 615.2830 (MH^+), found 615.2829.

N-Boc-O-methyl-2'-nitrocadabicine 188

N^2-t-Butoxycarbonyl-N^3-4-fluoro-3-nitrocinnamoyl-N^1-3-hydroxy-4-methoxycinnamoylspermidine **187** (49 mg, 0.081 mmol) and CsF (37 mg, 0.024 mmol)were combined in anhydrous DMF (14 mL) and were stirred for 18 h. H_2O (30 mL) was added to the reaction mixture and this solution was washed with EtOAc (3 x 30 mL). The combined organic washes were dried ($MgSO_4$) and evaporated *in vacuo*. The residue was purified by RP-HPLC (1:4 aq. TFA (0.1 %)/MeOH, λ=250 nm) to yield the product **188** as a buff foam (34 mg, 71 %). ^{1}H-NMR ($CDCl_3$) δ 1.43 (9 H, s, $C(C\underline{H}_3)_3$), 1.55 (4 H, bs, 2 x $CH_2C\underline{H}_2CH_2$), 1.73 (2 H, bs, $CH_2C\underline{H}_2CH_2$), 3.1-3.48 (8 H, 4 x $C\underline{H}_2N$), 3.90 (3 H, s, $OC\underline{H}_3$), 6.30 (1 H, d, J=16), 6.42 (1 H, d, J=16), 6.78 (1 H, d, J=8), 6.85-6.97 (1 H, bm), 7.08 (1 H, bs), 7.49 (1 H, bd, J=16), 7.53-7.64 (2 H, bm), 7.70 (1 H, bd, J=8), 7.94 (1 H, d, J=16); FAB-MS m/z 595 (MH^+), $C_{31}H_{38}N_4O_8$ requires 594; FAB-HRMS calcd. 595.2768 (MH^+), found 595.2793.

2'-Nitrocadabicine 189

O-Methyl-2'-nitrocadabicine (20 mg; 0.040 mmol) was dissolved in anhyd. CH_2Cl_2 (1 mL) and the solution was cooled to -78 °C. BBr_3 (11 mg; 0.044 mmol) was added and the solution was stirred for 3 h. The reaction mixture was diluted with CH_2Cl_2 (20 mL) and was then extracted with aq. 1 M NaOH (3 x 20 mL). The organic layer was dried ($MgSO_4$) and evaporated *in vacuo*. The residue was purified by flash column chromatography on silica gel eluting with 10 % MeOH/CH_2Cl_2 to yield the title compound **189** as a buff coloured gum (11 mg; 60 %).^{1}H-NMR ($CDCl_3$) δ 1.49 (4 H, bs, 2 x $CH_2C\underline{H}_2CH_2$), 1.68 (2 H, bs, $CH_2C\underline{H}_2CH_2$), 2.85-2.97 (4 H, 2 x $C\underline{H}_2N$), 3.09-3.33 (4 H, 2 x $C\underline{H}_2N$), 6.28 (1 H, d, J=16), 6.40 (1 H, d, J=16), 6.76 (1 H, d, J=8), 6.80-6.95 (1 H, m), 7.08 (1 H, bs), 7.47 (1 H, d, J=16), 7.52-7.67 (2 H, bm), 7.70 (1 H, bd, J=8), 7.96

(1 H, d, J=16); FAB-MS m/z 481 (MH^+), $C_{25}H_{28}N_4O_6$ requires 480; FAB-HRMS calcd. 481.2087 (MH^+), found 481.2099.

N^2, N^3-di-(*t*-Butoxycarbonyl)spermine 192 (O'Sullivan *et al.*, 1997)

Spermine **1** (2.00 g, 9.88 mmol) was dissolved in THF (10 mL) then ethyl trifluoro-acetate (2.4 mL, 19.7 mmol) was added over 5 min. The reaction mixture was stirred for 1 h then Boc anhydride (3.23 g, 14.8 mmol) was added. The reaction mixture was stirred for a further 18 h then conc. aq. NH_3 was added to the reaction mixture until the pH was >11. The reaction mixture was stirred for 24 h until TLC showed the reaction was complete. The reaction mixture was evaporated *in vacuo* and the residue was purified by flash column chromatography on silica gel eluting with CH_2Cl_2/MeOH/ conc. aq. NH_3 (15:10:1) to yield the title compound **192** as a colourless oil (3.14 g, 55 %). ^{1}H-NMR (CD_3Cl) δ 1.43 (18H, s, 2 x $C\underline{H}_3)_3$), 1.44-1.52 (4H, m, 2 x $CH_2C\underline{H}_2CH_2$), 1.65-1.70 (4H, m, 2 x $CH_2C\underline{H}_2CH_2$), 2.65-2.79 (4 H, m, 2 x $C\underline{H}_2NH_2$), 3.13-3.30 (8 H, m, 4 x $C\underline{H}_2N$); ^{13}C-NMR δ; 25.2, 28.0 ($\underline{C}H_3$), 28.5, 37.5, 43.4, 46.0, 79.9 ($C(C\underline{H}_3)_3$), 161.8 ($\underline{C}$=O); FAB-MS m/z 403 (MH^+) $C_{20}H_{42}N_4O_4$ requires 402.

N^1-3-Hydroxy-4-methoxycinnamoyl-N^2,N^3-di *t*-butoxycarbonylspermine (used to prepare **193**)

3-Hydroxy-4-methoxycinnamic acid (241 mg, 1.24 mmol) was dissolved in THF (7 mL). DCC (384 mg, 1.87 mmol), 2-mercaptothiazolidine (163 mg, 1.37 mmol) and DMAP (1 mg) were added and the reaction mixture was stirred for 1 h. The white precipitate was removed from the yellow solution by filtration. N^2, N^3-Di-(*t*-butoxycarbonyl)spermine (1.0 g, 2.49 mmol) was dissolved in THF (30 mL) and the yellow filtrate was added in one portion. The reaction mixture was stirred for 10 h and

was then evaporated *in vacuo*. The residue was purified by flash column chromatography on silica gel eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (50:10:1) to yield the title compound as a white wax (424 mg, 59 %). ^{1}H-NMR ($CDCl_3$) δ 1.33-1.57 (2H, m, 3 x C($C\underline{H}_3$)$_3$, 2 x $CH_2C\underline{H}_2CH_2$), 1.63-1.82 (4H, m, 2 x $CH_2C\underline{H}_2CH_2$), 2.77 (2H, bt, *J*=7, $C\underline{H}_2NH_2$), 3.05-3.42 (10 H, m, 2 x $C\underline{H}_2NBocC\underline{H}_2$, $C\underline{H}_2NC{=}O$), 3.87 (3 H, s, $OC\underline{H}_3$), 6.35 (1H, d, *J*=16, $C^{2'}\underline{H}$), 6.80 (1 H, d, *J*=8, $C^6\underline{H}$), 6.95 (1 H, bd, *J*=8, $C^5\underline{H}$), 7.10 (1 H, bs, $C^2\underline{H}$), 7.50 (1 H, d, *J*=16, $C^{1'}\underline{H}$); ^{13}C-NMR δ; FAB-MS *m/z* 579 (MH^+) $C_{30}H_{50}N_4O_7$ requires 578.

N^3-4-Fluoro-3-nitrocinnamoyl-N^1-3-hydroxy-4-methoxycinnamoyl-N^2,N^3-di-*t*-butoxycarbonylspermine 193

4-Fluoro-3-nitrocinnamic acid **176** (42 mg, 0.20 mmol) was dissolved in THF (5 mL). DCC (61 mg, 0.30 mmol), 2-mercaptothiazolidine (26 mg, 0.22 mmol) and DMAP (1 mg) were added and the reaction mixture was stirred for 1 h. The white precipitate was removed from the yellow solution by filtration. N^1-3-hydroxy-4-methoxycinnamoyl-N^2,N^3-di *t*-butoxycarbonylspermine (114 mg, 0.20 mmol) was dissolved in THF (5 mL) and was added to the yellow filtrate. The reaction mixture was stirred for 10 h and was then evaporated *in vacuo*. The residue was purified by flash column chromatography on silica gel eluting with 5 % MeOH /CH_2Cl_2 to yield the title compound **193** as a buff coloured foam (111 mg, 73 %). ^{1}H-NMR ($CDCl_3$) δ 1.50 (18 H, s, 2 x C($C\underline{H}_3$)$_3$), 1.60-2.00 (8 H, m, 4 x $CH_2C\underline{H}_2CH_2$), 3.03-3.43 (12 H, 6 x $C\underline{H}_2N$), 3.87 (3 H, s, $OC\underline{H}_3$), 6.31 (1 H, d, *J*=16), 6.47 (1 H, d, *J*=16), 6.80 (1 H, d, *J*=8), 6.87-6.99 (1 H, m), 7.10 (1 H, bs), 7.46 (1 H, d, *J*=16), 7.55-7.78 (3 H, m), 7.93 (1 H, d, *J*=16); ^{13}C-NMR δ FAB-MS *m/z* 772 (MH^+), $C_{39}H_{54}FN_5O_{10}$ requires 771; FAB-HRMS calcd. 772.3933 (MH^+), found 772.3930.

Boc-protected 28-membered macrocycle 194

N^3-4-Fluoro-3-nitrocinnamoyl-N^1-3-hydroxy-4-methoxycinnamoyl-N^2,N^3-di-*t*-butoxycarbonylspermine (61 mg, 0.079 mmol), 18-crown-6 (208 mg, 0.79 mmol) and anhydrous K_2CO_3 (33 mg, 0.24 mmol)were combined in anhydrous DMSO (14 mL) and were stirred for 5 h at 50 °C. H_2O (30 mL) was added to the reaction mixture and this solution was washed with EtOAc (3 x 30 mL). The combined organic washes were dried ($MgSO_4$) and evaporated *in vacuo*. The residue was purified by RP-HPLC (1:4 aq. TFA (0.1 %)/MeOH, λ=250 nm) to yield the product as a buff foam (39 mg, 66 %). ^{1}H-NMR ($CDCl_3$) δ 1.40 (9 H, s, $C(C\underline{H}_3)_3$), 1.53-1.80 (8 H, bs, 4 x $CH_2C\underline{H}_2CH_2$), 2.98-3.34 (12 H, 6 x $C\underline{H}_2N$), 3.83 (3 H, s, $OC\underline{H}_3$), 6.23 (1 H, d, *J*=16), 6.32 (1 H, d, *J*=16), 6.73 (1 H, d, *J*=8), 6.91 (1 H, bd, *J*=9), 7.03 (1 H, bs), 7.20-7.33 (1 H, bm), 7.43 (1 H, bd, *J*=16), 7.48-7.60 (1 H, bm), 7.67 (1 H, bd, *J*=8), 7.85 (1 H, d, *J*=16); FAB-MS *m/z* 752 (MH^+), $C_{39}H_{53}N_5O_{10}$ requires 751; FAB-HRMS calcd. 752.3871 (MH^+), found 752.3895.

28-Membered macrocycle 195

Compound **194** (20 mg, 0.067 mmol) was stirred in trifluoroacetic acid (1 mL) and CH_2Cl_2 (1 mL) at 0 °C for 45 min. The solvents were removed *in vacuo*, as an azeotrope with water and MeOH. The residue was purified by flash column chromatography eluting with CH_2Cl_2/MeOH/conc. aq. NH_3 (100:10:1) to yield compound **195** as a colourless glass (12 mg, 80 %). ^{1}H-NMR ($CDCl_3$) δ 1.57-1.75 (8 H, bs, 4 x $CH_2C\underline{H}_2CH_2$), 2.98-3.34 (12 H, 6 x $C\underline{H}_2N$), 3.81 (3 H, s, $OC\underline{H}_3$), 6.22 (1 H, d, *J*=16), 6.32 (1 H, d, *J*=16), 6.75 (1 H, d, *J*=8), 6.91 (1 H, bd, *J*=9), 7.03 (1 H, bs), 7.20-7.33 (1 H, bm), 7.43 (1 H, bd, *J*=16), 7.48-7.57 (1 H, bm), 7.66 (1 H, bd, *J*=8), 7.80 (1 H, d, *J*=16); FAB-MS *m/z* 552 (MH^+), $C_{29}H_{37}N_5O_6$, requires 551.

Chapter 7

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Appendix

Part of the work described in this thesis has been published in the following publications:

Carrington, S.; Qarawi, M. A.; Blagbrough, I. S.; Moss, S. H.; Pouton, C. W. Inhibition of the Growth of B16 Murine Melanoma Cells by Novel Spermine Analogues. *Pharm. Sci.* **1996**, *2*, 25-27.

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Inhibition of Growth of B16 Murine Melanoma Cells by Novel Spermine Analogues

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Abstract

To develop new cytotoxins, which could find use as anti-cancer agents, polyamine conjugates were synthesized containing spermine and an anthracene or acridine unit. Spermine is known to groove-bind to DNA; anthracene and acridine are known to intercalate. It was hoped that these polyamine–polyaromatic conjugates would use both modes of binding.

Studies of growth inhibition of B16 murine melanoma cells showed the conjuates to be more effective than either spermine, anthracene-9-carboxylic acid or acridine-9-carboxylic acid, and of the conjugates, the acridine derivative showed greatest activity.

Polyamines, such as spermine (1) and spermidine (2) (Fig. 1), occur naturally in cells in at least μM concentrations (Tabor & Tabor 1984). Although their precise functions are not fully understood, they are known to be involved with cell growth and replication and are thought to play a role in the stability of chromatin structure (Heby & Persson 1990; Basu et al 1992). Polyamines are known to have anti-tumour activity which is attributed to either general depletion of polyamine pools, down-regulation of enzymes such as ornithine decarboxylase and spermine/spermidine-N^1-acetyltransferase (Bernacki et al 1992) or DNA binding and interference with DNA transcription. At physiological pH, most naturally occurring polyamines are fully protonated, the pK_a values for spermine are 11·50, 10·95, 9·79 and 8·90 (Takeda et al 1983; Usherwood & Blagbrough 1989). These positive charges interact with the negative charges on the sugar-phosphate backbone of DNA causing them to bind from either the major or minor groove (Rodger et al 1995). This electrostatic bonding can be further supplemented by hydrophobic van der Waals' interactions between methylene groups in the polyamine chain and methyl groups on thymine bases in thymidine.

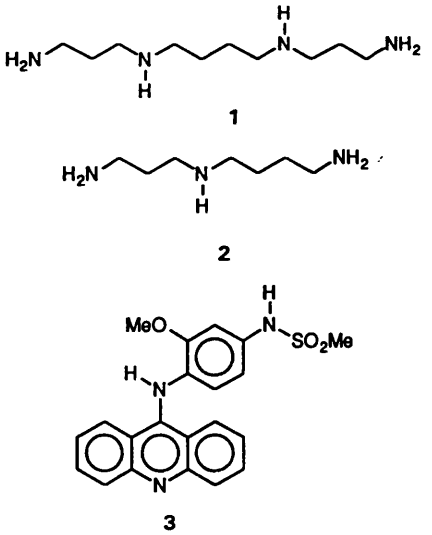

FIG. 1. Structures of spermine (1), spermidine (2) and amsacrine (3).

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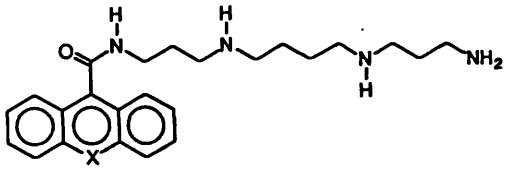
DNA intercalators incorporate planar aromatic ring systems which intercalate between the heterocyclic base pairs, perpendicular to the axis of the double helix (Wilson 1990). Intercalators bind by interaction between their π-orbitals and those in the DNA bases. The 9-aminoacridine derivative amsacrine (3, Fig. 1) is an intercalator which has been shown to have anti-cancer activity, currently finding use in the clinic to treat acute leukemia (Baguley 1991). Structure–activity relationship studies of a series of analogues have shown that the strength of intercalation closely correlates to the activity as an anti-tumour agent (Ferguson & Baguley 1981).

To obtain compounds with enhanced cytotoxicity, we designed the synthesis of conjugates with bifunctional modes of binding to DNA. Studies of amsacrine and other ligands with substituents on the aromatic ring system have shown that they can protrude into one of the DNA grooves. If this substituent is a known groove-binder, such as spermine (1), then spectroscopy and computer modelling have shown that it is possible to obtain molecules simultaneously displaying both modes of binding (Adlam et al 1994; Rodger et al 1994, 1995). This dual interaction should strengthen the association between ligand and DNA, and therefore increase the cytotoxicity compared to that shown by either the groove-binder or the intercalator alone. Our target compounds (Fig. 2) consisted of either an anthracene (4) or an acridine (5) unit linked to spermine via an amide bond at position 9. These conjugates were synthesized, purified and then screened against B16 murine melanoma cells for cytotoxicty. For comparison, spermine (1), anthracene-9-carboxylic acid and the pyridine analogue acridine-9-carboxylic acid, and mixtures of spermine with these acids (1:1, w/w) were also screened.

Materials and Methods

Chemistry

The synthetic conjugates were prepared by coupling spermine with either anthracene or acridine-9-carboxylic



4 X = CH
5 X = N

FIG. 2. Structures of the two synthetic conjugate target compounds.

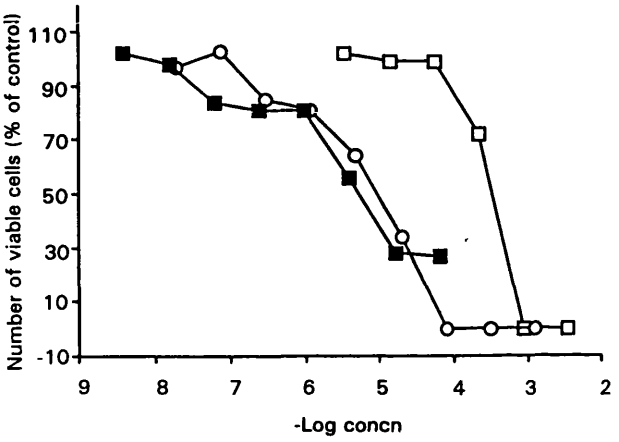


FIG. 3. Activity of spermine (1) (□) and the two synthetic conjugates 4 (○) and 5 (■) in the MTT assay.

acids. Initially, the acid was coupled using dicyclohexylcarbodiimide (DCC) and a catalytic amount (0·05 equivalents) of hydroxybenzotriazole (HOBt) to spermine with one primary amine protected with a *t*-butoxycarbonyl (BOC) group. These couplings gave poor yields which were found to be due to side reactions with the secondary amine groups. This problem was overcome by using spermine which had been protected with benzyloxycarbonyl (Z) groups on both secondary amines and one of the primary amines.

Therefore, spermine was first reacted with BOC anhydride in dichloromethane in the ratio three equivalents of spermine to one of anhydride. The N^1-mono-BOC protected spermine was isolated in 27% yield by flash column chromatography on silica gel (eluant 10:4:1 CH_2Cl_2–methanol–conc. NH_4OH, v/v/v). Reaction with benzyl chloroformate in aqueous sodium hydroxide solution gave 94% tri-Z-mono-BOC spermine after work-up. Stirring with trifluoroacetic acid at 0°C for 45 min cleaved the BOC group yielding tri-Z spermine in 94% yield. This was coupled to anthracene-9-carboxylic acid and acridine-9-carboxylic acid using DCC and a catalytic amount of HOBt (0·05 equivalents) in dichloromethane and dimethylformamide, respectively. The protected intermediates were recovered in yields of 70 and 60%, respectively, after chromatography. The target compounds were obtained in 90% yield after catalytic hydrogenolysis over 10% palladium on carbon.

MTT assay

The trial compounds were screened in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as reported by Mosmann (1983).

B16 murine melanoma cells were incubated in RPMI medium containing test compounds at 37°C for 48 h (4000 cells/well in a 96-well plate). The plate was washed and then incubated with 1 mg mL^{-1} MTT in serum-free RPMI medium for 3 h. The blue formazan crystals produced by viable cells were dissolved in dimethylsulphoxide to allow assay using a microplate spectrophotometer at 540 nm (test) and 690 nm (background). Formazan generation was a linear function of the number of viable cells present, allowing the estimation of the EC50 for growth inhibition.

Results and Discussion

The results from the assays are shown in Table 1 and Fig. 3. The anthracene-9-carboxylic acid and acridine-9-carboxylic acid both had negligible effects. Spermine (1) inhibited cell growth with an EC50 of 3×10^{-4} M and simply mixing spermine with the aromatic acids showed no increase in potency over spermine alone. The conjugate (4) containing the anthracene unit showed inhibition with an EC50 of 2×10^{-5} M, an increase in potency of an order of magnitude over spermine, whilst the acridine analogue (5) had an EC50 of 6×10^{-6} M, almost a fiftyfold increase in potency over spermine. These results show that the synthetic conjugates combining both an intercalator and groove-binder show greater cytotoxicity than compounds which exhibit just one of these modes of binding. Co-administration of a polyamine with an intercalator showed no enhancement of potency, demonstrating the necessity to link physically the two moieties in order to give bifunctional binders. Such bifunctional DNA-ligands inhibit the growth of B16 murine melanoma cells and are therefore novel spermine analogues which offer a new lead in the design of cytotoxic polyamines with anti-cancer activity.

Acknowledgements

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Table 1. Growth inhibition of B16 murine melanoma cells as determined by the MTT assay.

Compound	EC50 (M) (mean ± s.d.)	-log EC50n	
Spermine (1)	$2{\cdot}9 (\pm 1{\cdot}5) \times 10^{-4}$	3·5	2
Anthracene-9-carboxylic acid	$> 10^{-3}$	< 3	3
Acridine-9-carboxylic acid	$> 10^{-3}$	< 3	3
Anthracene-9-carboxylic acid and spermine (1:1, w/w)	$2{\cdot}2 (\pm 0{\cdot}6) \times 10^{-4}$	3·7	3
4	$1{\cdot}8 (\pm 0{\cdot}3) \times 10^{-5}$	4·8	2
5	$6{\cdot}2 (\pm 2{\cdot}3) \times 10^{-6}$	5·2	3

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Pharmaceutical Sciences 1997, 3: 223–233

Polyamines and Polyamine Amides as Potent Selective Receptor Probes, Novel Therapeutic Lead Compounds and Synthetic Vectors in Gene Therapy

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Abstract

The family of polyamines and polyamine amides, especially unsymmetrical synthetic members, is critically assessed with respect to chemical structure and pharmacological activity. Naturally occurring polyamines (and diamines) and mono- (and di-) acylated polyamines (polyamine amides) are blockers of cation channels that are receptor- or voltage-gated. Such compounds are leads for the design of novel therapeutic agents. Furthermore, polyamines and polyamine amides are templates for the design of synthetic vectors with potential application in gene therapy.

Natural di- and polyamines, spider and wasp venom polyamine amide toxins and their analogues, and totally synthetic polyamines, are potent cation-channel blockers with uses as selective receptor probes for nicotinic acetylcholine and glutamate (NMDA and non-NMDA) receptors, sodium and calcium channels. Therefore, as receptor probes, they may help us to understand the molecular mechanisms of neurodegeneration, and ultimately to design drugs for the treatment of neurodegenerative diseases, especially stroke. Polyamine conjugates are also novel therapeutic lead compounds for possible treatments of cancer, diarrhoea, malaria and haemochromatosis (β-thalassaemia). The metal chelating properties of (poly-) ethylenediamines have led to their incorporation in ion chelators which are synthetic RNase and DNase enzymes. Synthetic polyamines and polyamine amides have potential as novel vectors in gene delivery. Such compounds can condense DNA to form toroidal particles which may be incorporated in a non-viral gene delivery system. The applications of polylysine, polyethylenimine, Starburst polyamidoamine dendrimers, Transfectam (DOGS), cholic acid, and cholesterol conjugates to gene therapy are compared as a function of structure and pKa.

This assessment of polyamines and polyamine amides stresses the basicity of the amine functional groups. The pKa's of these functionalities are a major determinant in their binding to biological macromolecules. Selectivity of pharmacological action also encompasses contributions from solution conformation and lipophilicity as well as amine pKa. The use of these compounds as leads for the design of novel therapeutics or gene medicines is demonstrated to be practical as well as theoretically possible.

Naturally occurring polyamines, such as the tetraamine spermine (**1**) and the triamine spermidine (**2**) (Figure 1) occur in cells at micromolar concentrations, and may even rise to millimolar levels in certain cancer cells (Tabor & Tabor 1984). The biosynthetic building blocks for these and closely related polyamines are the α-amino acids ornithine and lysine, affording the diamines putrescine (**3**) (1,4-diaminobutane) and cadaverine (**4**) (1,5-diaminopentane), respectively (Figure 1). In recent years, we have established that polyamines, and the new class of cation-channel blocking agents polyamine amides, derived from analogy with the low molecular weight fraction of the venoms of certain spiders and a parasitic wasp, have potential as potent, selective receptor probes and even as novel therapeutic lead compounds in the design of antitumour agents. Other workers, especially and Bergeron et al (1987, 1989), have addressed the usefulness of polyamines in cancer chemotherapy. In more recent studies, polyamines have been

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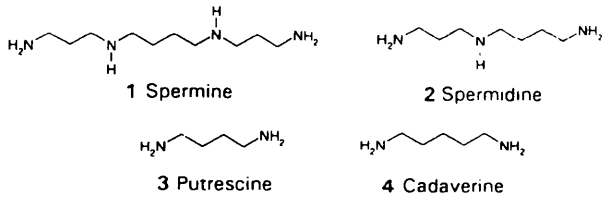

Figure 1. Structures of physiological polyamines and diamines.

identified as novel leads for the design of anti-diarrhoeal agents and antimalarials, and as ion chelators. Furthermore, the possibility of using poly-amines (especially the dimers to hexamers of ethyl-enediamine) for the design of specific synthetic RNase or DNase enzymes has been addressed in elegant studies by Yoshinari et al (1991).

We also consider the potential for polyamines, in an extension of one of their possible in-vivo roles, in maintaining DNA condensation working with or without histones to stabilize the three-dimensional structure of polynucleic acids. Thus, polyamines and polyamine amides display many of the ideal properties of synthetic vectors for polynucleotide delivery in gene therapy. Recent improvements in the use of polylysine in DNA condensation and cell transfection have come with the design of unsymmetrical polyamine amides and the application of synthetic polyamines. Polyethylenimine and Starburst polyamidoamine dendrimers are two such polyamine based gene-delivery systems. Transfectam (DOGS) is an unsymmetrical glycine–spermine conjugate, designed and prepared by Behr et al (1989). It incorporates two long lipid chains for bilayer formation. DOGS is the parent member of lipopolyamine gene-delivery vectors, forming compact particles which are not cationic liposomes. By analogy, cholesterol and cholic acid conjugated unsymmetrical polyamine amides have recently been reported as synthetic vectors for gene therapy.

Ion-channel blockers

Spermine (**1**) is found in the venom of certain spiders where it accompanies a range of small-molecule phenols (Fischer & Bohn 1957; Gilbo & Coles 1964). The venom of a solitary parasitic wasp (*Philanthus triangulum*) contains thermospermine (a regioisomer of spermine) conjugated to L-tyrosine in PhTX-4.3.3 (**5**) (Figure 2). This unsymmetrical polyamine amide is essentially equipotent with synthetic spermine-containing analogue PhTX-3.4.3 (**6**). These polyamine amides (philanthotoxins) are potent blockers of the cation channels gated exogenously by nicotine and endogenously by acetylcholine. That they are cation-channel blockers is not surprising when one considers their structures (Figure 2), essentially fully protonated at physiological pH. By comparison, the pKa's of spermine (**1**) and spermidine (**2**) are 11·50, 10·95, 9·79, 8·90 and 11·56, 10·80, 9·52, respectively (Takeda et al 1983). The corresponding data for PhTX-3.4.3 (**6**) are 11·4, 10·4, 9·5 and 8·5, although the measured pKa of 9·5 also accounts for the phenolic functional group, and therefore an increase in acidity from pH 10·4 to 9·5 finds both the secondary amine nearer to the tyrosine residue, and the phenoxide of tyrosine gaining protons (Jaroszewski et al 1996). The pKa of phenol is 10·0, and the pKa's of tyrosine are 10·07 and 9·11 (and 2·20).

Many polyamine amides have now been isolated from the venom of certain spiders, (reviews by Blagbrough & Usherwood 1992; Schäfer et al 1994) and characterized pharmacologically (reviews by Usherwood & Blagbrough 1991; Carter 1995; Mueller et al 1995). These low molecular weight toxins (argiotoxins) (**7–12**, Figure 3) are potent selective non-competitive antagonists of glutamate receptors, blocking the cation-selective channels associated with this excitatory α-amino acid (both NMDA and non-NMDA glutamate receptors). Therefore, they have potential as pharmacological probes and as lead compounds for the design of drugs to treat neurodegeneration, especially stroke (Parks et al 1991; Blagbrough & Usherwood 1992; Carter 1995). However, unsymmetrical polyamine amides require complete syntheses for sufficient material to be available for detailed pharmacological characterization.

The novel polyamine FTX-3.3 (**13**) (Blagbrough & Moya 1994) and the polyamine amide sFTX-3·3 (**14**) (Moya & Blagbrough 1994) are important pharmacological tools (Figure 4) for modulation of voltage-sensitive calcium channels (VSCC). These polyamines block VSCC with differential inhibi-

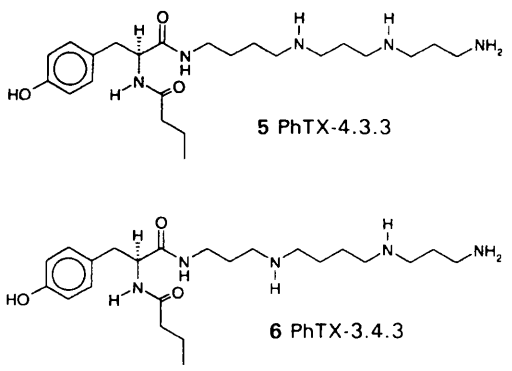

Figure 2. Structures of polyamine amide wasp toxin PhTX-4.3.3 (**5**) and its essentially equipotent analogue PhTX-3.4.3 (**6**).

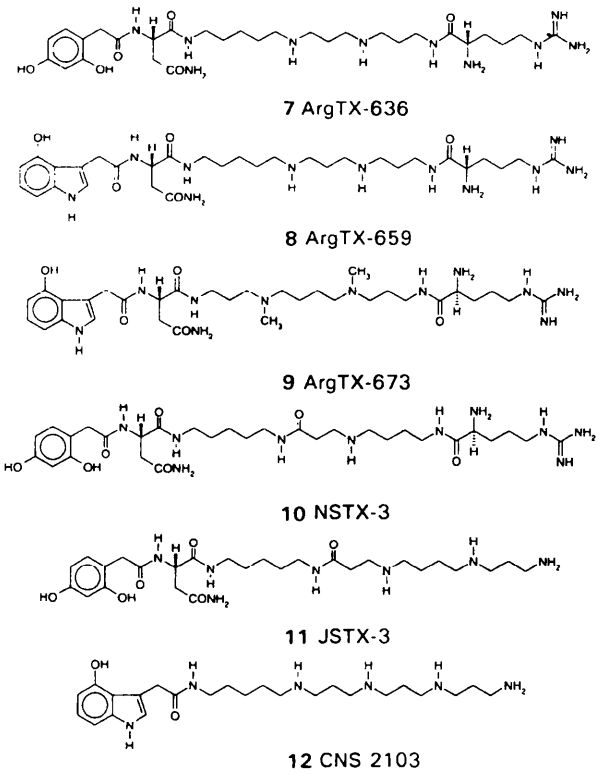

Figure 3. Structures of polyamine amide spider toxins.

tion in mature rat cerebellar Purkinje cells (Dupere et al 1996) and antagonize P-, N- and L-type VSCC in a voltage-dependent manner (Norris et al 1996). Other pharmacological evidence for cation-channel blockage comes from one endogenous role of cytosolic spermine (**1**) and spermidine (**2**) as gating molecules for inward rectifying potassium channels (Ficker et al 1994; Lopatin et al 1994).

Cancer

There is ever increasing realization of the biological effects of polyamines, particularly in cellular processes, including growth and replication (Heby & Persson 1990). Thus, it is not surprising that polyamine conjugates continue to be the focus of significant attention as potential anticancer agents.

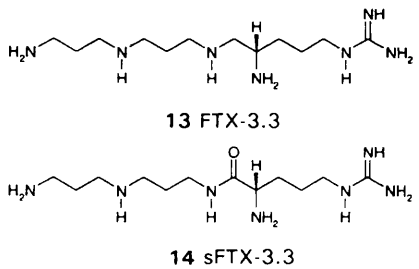

Figure 4. Structures of synthetic polyamines which modulate voltage-sensitive calcium channels.

There is a polyamine transporter which specifically mediates the uptake of extracellular polyamines into cells (Seiler & Dezeure 1990), and rapidly dividing tumour cells require large quantities of polyamines. Consequently, this polyamine transporter is up-regulated in tumour cells moreso than in normal cells (Seiler et al 1990). Polyamines groove-bind to DNA from either the major or the minor groove (Rodger et al 1994, 1995) and it is thought that endogenous polyamines also affect chromatin stability and structure (Basu et al 1992). The biosynthetic pathway of the common physiological di- and polyamines, putrescine (**3**), cadaverine (**4**), spermidine (**2**) and spermine (**1**) is well characterized (reviews by Tabor & Tabor 1984; Marton & Pegg 1995). Indeed, inhibitors have been synthesized for some of the key enzymes (Guo et al 1995; Pegg et al 1995). Taking these three aspects into account when designing polyamine based anticancer agents, there exists a potential uptake mechanism with selectivity for cancer cells (Cohen & Smith 1990) and two possible modes of cytotoxicity. This toxicity may be mediated either by DNA binding and hence disruption of transcription (Feuerstein et al 1990), or by interference with polyamine biosynthetic pathways thereby modulating the cellular concentrations of endogenous polyamines.

To date, some of the simplest and most effective synthetic polyamines to show anticancer activity have been developed by Porter, Bergeron and their co-workers. They initially found activity with spermidine and spermine analogues which are *N*-alkylated (Porter et al 1982, 1985). Further studies showed the best analogues to be tetra-amines which have been *bis*-ethylated on the terminal, primary amines (e.g. **15**, **16** and **17**) (Bergeron et al 1987, Porter et al 1987) (Figure 5). These compounds are

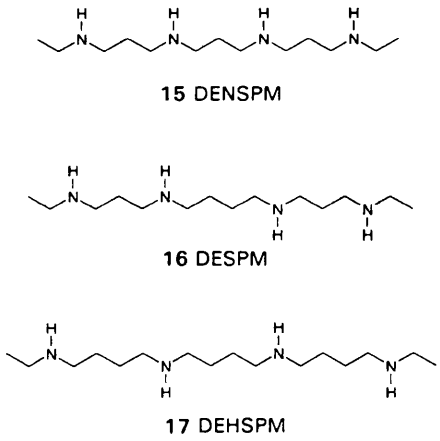

Figure 5. Structures of synthetic antiproliferative polyamines DENSPM (**15**), DESPM (**16**) and DEHSPM (**17**).

recognized and taken into cells by the polyamine transporter. Once inside the cells, they deplete intracellular polyamine pools by down-regulating the enzyme ornithine decarboxylase (ODC), the first enzyme in the polyamine synthesis pathway, and up-regulating the spermine–spermidine N^1-acetyltransferase (SSAT) enzyme which works in the back conversion pathway (Bergeron et al 1989). The synthetic analogues were found to replace the physiological polyamines and, over a 24-h period in-vitro, it was found that the total amount of polyamine normalized and the nitrogen content of each molecule (i.e. 3 in spermidine, 4 in spermine) remained constant. The cytotoxic effects of the analogues DENSPM (**15**), DESPM (**16**) and DEHSPM (**17**) in in-vitro cultures of L1210 cells, over 96 h, were 1·3 μM, 0·2 μM and 0·06 μM respectively. Investigation into their mode of action showed a significant variation in their ability to compete with native polyamines for cellular uptake, but in time it was found that they reached comparable concentrations in the cells and had similar depleting effects on endogenous polyamine pools. As the analogues show different cytotoxic effects, the displayed cytotoxicity might be partly due to some site-specific interactions not involved in polyamine biosynthesis. Investigations are ongoing both into the mechanism of action of these compounds and into their clinical use.

Another approach to the development of antitumour compounds is the covalent linking of cytotoxic agents, whose activity is mediated through direct interaction with DNA, to a polyamine. The resulting conjugate will be transported into the cell through the polyamine transport mechanism (if recognized) and the polyamine should further aid DNA binding of the cytotoxic component at its DNA target site. We have been linking polyaromatic anthracene and acridine moieties to spermine (Carrington et al 1996). Acridine derivatives, especially 9-aminoacridines, show pronounced antitumour activity. Extensive research into structure–activity relationships culminated in the antileukaemic drug amsacrine (Denny et al 1983). The activity of these compounds is due to their ability to bind to DNA through intercalation, resulting in disruption of DNA transcription. Initially, the N^1 position of spermine was bound to the 9 position of anthracene through an amide bond. The interaction of the resulting conjugate with DNA was then investigated by linear and circular dichroism, and normal absorption techniques (Adlam et al 1994; Rodger et al 1994). These data were supported by dynamic computer modelling simulations of the conjugate in the presence of a strand of DNA (Adlam et al 1994; Rodger et al 1995). One of the outcomes of these experiments was the conclusion that this conjugate can bind in a bifunctional manner with the polyamine in the groove, and the anthracene intercalating between the base pairs. In-vitro structure–activity studies on a series of analogues (**18–22**) (Figure 6), on B16 murine melanoma cells, have shown that the best activity is achieved by attaching spermine to acridine via an aniline (Qarawi et al 1997). The most potent cytotoxin in this series, synthesized to date, also has a 5-carbon spacer derived from 5-aminovaleric acid between the acridine and spermine moieties (**21**) which may confer a region of flexibility between the two bonding regions.

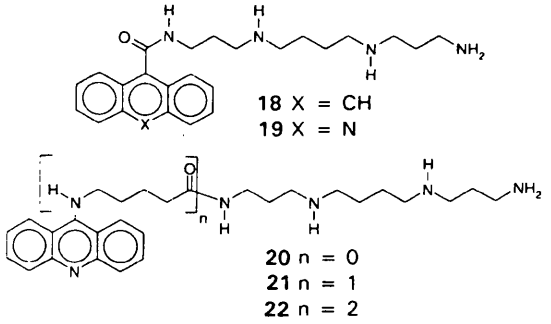

Figure 6. Structures of cytotoxic synthetic conjugates of polyamines with tricyclic aromatics.

Chlorambucil is a nitrogen mustard containing compound which is used to treat a number of cancers. Its mechanism of action is DNA alkylation and hence cross-linking of DNA strands. Chlorambucil has been conjugated to both spermidine (**2**) and spermine (**1**), resulting in DNA cross-linkers with the potential to carry up to 3 (**23**) or 4 (**24**) positive charges at physiological pH (Cohen et al 1992, Cullis et al 1995) (Figure 7). In-vitro experiments have established that these polyamine conjugates are recognized by cellular uptake systems—the spermidine conjugate displayed ~35 times more cytotoxicity than chlorambucil alone. These conjugates alkylate DNA in the same positions as chlorambucil indicating that the polyamine moiety does not affect the mechanism of alkylation. However, in-vivo studies did not display the high level of activity predicted by in-vitro assays (Cullis et al 1995). A more promising approach to the design and synthesis of polyamines containing a reactive functional group capable of DNA alkylation has recently been described (Li et al 1996). Spermidine and spermine analogues (e.g. **25**) were prepared with the primary amines replaced by aziridine functional groups (Figure 7) to give a *bis*-alkylating agent bound to a polyamine backbone. Studies showed these compounds to be transported into cells and to cross-link DNA. The in-vivo

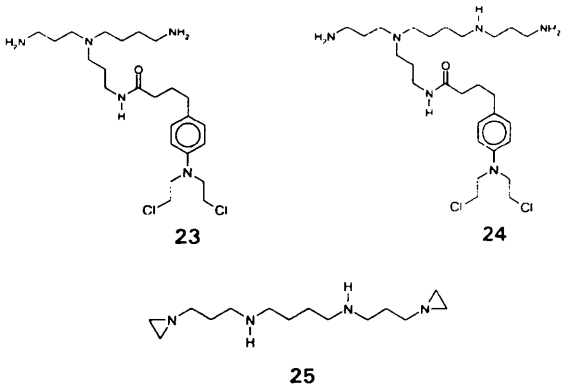

Figure 7. Structures of polyamine-containing compounds which cross-link DNA.

activity of spermine analogue **25** was comparable with that shown by other *bis*-alkylating agents in the same assay, making it a lead compound with the potential for further development.

Diarrhoea

Tabor & Tabor (1964) reported that relatively high concentrations of spermine (**1**) and spermidine (**2**) are found in the gastrointestinal tract. The physiological activity of polyamines in the gut was subsequently characterized with initial investigations carried out with polymers derived from ethylenimine, commonly available polyamine-containing compounds with a variety of industrial uses (Melamed et al 1977). Branched-chain polymers were shown to inhibit gastric emptying in rats, while linear structures had negligible physiological activity. It was thought that polyamines might find use as appetite suppressants or in prolonging the action of orally administered drugs. However, further experimentation involving oral administration to dogs resulted in a severe retching response (Tansy et al 1977). Investigations were also carried out with small-molecule polyamines, for example, spermine (**1**), spermidine (**2**) and synthetic close analogues (Belair et al 1981). The naturally occurring polyamines were found to have a profound effect on gastric emptying, while synthetic analogues had significantly lower activities.

In HIV-related infections, there is a frequent occurrence of serious diarrhoea, either as a result of infection or as a side effect from certain drugs. So far this has been difficult to treat, existing drugs giving only a partial response and a high relapse rate. A new approach to treatment, developed by Bergeron et al (1996), has been the use of synthetic polyamines to slow gut motility. After initial structure–activity assessment, DEHSPM (**17**) was found to show antidiarrhoeal activity in a castor-oil induced diarrhoea model in rats, and has now been used to treat patients in the clinic. The drawback with this compound is chronic toxicity associated with the accumulation of metabolites. In-vivo studies have shown that DEHSPM (**17**) is first metabolized by *N*-de-ethylation. Normally, the next stage in polyamine metabolism would be removal of the 3-aminopropyl moieties through the action of SSAT and polyamine oxidase. However, the remaining homospermine contains only 4-aminobutyl fragments which are not metabolized and therefore accumulate in the patient's tissues. This problem has been resolved by synthesizing compounds substituted with hydroxyl groups (on tetrahedral carbon atoms of *R*-configuration) (**26**) on methylenes located γ to the ethylated amines (Figure 8). These alcohol functional groups offer potential sites for enzymatic conjugation or oxidation leading to further metabolism and elimination. Studies showed that tetra-amine **26** retains its gastrointestinal activity and has significantly reduced chronic toxicity, although the exact mechanism of degradation is not known (Bergeron et al 1996). These results illustrate the way in which toxicity associated with polyamines can be reduced without the loss of therapeutic effect.

Malaria

Malaria continues to be a major health problem in the world today despite the progress which has been made in its treatment. Major problems are now related to strains of the malaria parasite which are resistant to chloroquine and other antimalarial drugs. This has led to a continuing search for drugs of different chemical classes and with new modes of action (Fairlamb & Cerami 1992). Much work has been carried out on the biosynthesis and function of diamines putrescine (**3**) and cadaverine (**4**), and polyamines spermidine (**2**) and spermine (**1**) in a number of human infective parasites (reviews by Tabor & Tabor 1984; Marton & Pegg 1995). α-Difluoromethylornithine (DFMO) (**27**) was identified as a potential therapeutic agent as it is known to be an inhibitor of ornithine decarboxylase, the enzyme which transforms ornithine to putrescine in the first stage in polyamine biosynthesis (Metcalf et al 1978; Bacchi et al 1987). In-vivo trials showed that DFMO inhibits the growth of parasites, but in

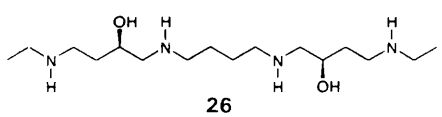

Figure 8. A substituted polyamine (**26**) for the treatment of diarrhoea.

some cases it fails to bring about a complete cure. A further approach involves synthetic polyamines which have previously found use in treating cancer (Bitonti et al 1989). Terminally *bis*-benzylated tetra-amines were initially identified as inhibiting the growth of the parasites *Plasmodium falciparum* and *Plasmodium berghi*. Further studies were carried out to investigate the optimum number of methylene groups required in the polyamine chain, as well as the effect of changing the benzyl substituents to moieties of similar size (e.g. cyclohexylmethyl and ring substituted benzyls) (Edwards et al 1991. As a result of testing both in-vitro and in-vivo (using a *P. berghi* infection in mice) a lead compound was chosen with terminal *N*-benzyl groups and a sequence of 3 then 7 then 3 methylene groups separating the four amines (**28**) (Figure 9). This tetra-amine is less potent than chloroquine yet more active than tetracycline, an antibiotic used to treat resistant strains of infection. On co-administration of tetra-amine **28** with DFMO (**27**), a complete cure was effected in the in-vivo mouse model. The mechanism of action of these compounds has not yet been fully investigated, but it is thought that they interfere with polyamine biosynthesis as well as possibly occupying key binding sites on DNA.

Iron chelation

In all forms of life, except for a few species of bacteria, iron plays an important role, principally in metabolic processes where the interconversion between the +2 and +3 oxidation states is used in a variety of redox proteins. In the environment, iron generally occurs at the ferric oxidation level which is largely insoluble and hence presents a problem to microorganisms. Microbes have solved this by generating iron chelator systems called siderophores which complex (sequester) ferric iron, allowing it to be accessed (Bergeron 1984). Many siderophores contain polyamine or polyamide moieties, for example, parabactin (**29**), agrobactin (**30**) and desferrioxamine B (**31**) (Figure 10). Phytosiderophores are low molecular weight ion-chelating compounds endogenous to plants. They facilitate iron solublization and transport in a manner analogous to the microbial siderophores. The phytosiderophore nicotianamine (**32**) (Scholz et al 1992; Matsuura et al 1994) is a triamine which contains a primary, a secondary and a tertiary amine functional groups. These polyamine based compounds are important as probes for the investigation of iron transport into various cells, the roles of iron in infection and, in their own right, as potential treatments for haemochromatosis (β-thalassaemia).

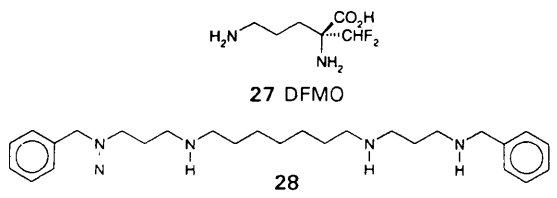

Figure 9. Structures of DFMO (**27**) and a tetra-amine (**28**) for the treatment of malaria.

RNase and DNase

The design and synthesis of synthetic catalysts which hydrolyse RNA with the aim of developing systems which mediate site-selective scission has recently been reported (Yoshinari et al 1991). Originally, the catalysts were based around transition metal chemistry, but recent developments have incorporated oligoamines, such as ethylenediamine, which will efficiently hydrolyse RNAs. In this cleavage, the mechanism of action involves an intramolecular acid–base co-operation between an ammonium cation and an uncharged amine. To make a site-selective RNA cleaving agent, ethylenediamine has been linked to a 19-mer piece of synthetic DNA (**33**) (Figure 11), designed so that the DNA sequence is complementary with the RNA sequence adjacent to the desired site for scission. Ethylenediamine is used here for its acidity, rather than as a metal-ion chelator. The pKa's of ethylenediamine are 9·2 and 6·5 (Yoshinari et al 1991), or 9·92 and 6·86 (Albert & Serjeant 1984). How-

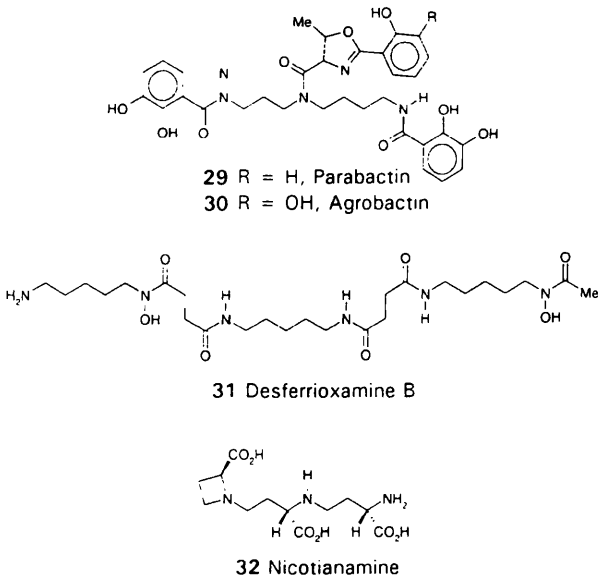

Figure 10. Structures of siderophores parabactin (**29**), agrobactin (**30**), desferrioxamine B (**31**) and nicotianamine (**32**).

ever, "that intramolecular co-operation between two amino residues plays a dominant role is strongly evidenced" with "remarkable acceleration of RNA hydrolysis by simple oligoamines as highly potent catalytic moieties for artificial ribonucleases" (Yoshinari et al 1991). DNA intercalators (anthraquinones) substituted with metal chelating moieties (ethylenediamines), and complexed to cupric ions, induce the chain cleavage of double stranded DNA (Ihara et al 1994). Other artificial ribonucleases have been designed on a polyamine or amine–imidazole template (Breslow 1995), with acridine used to effect RNA intercalation (Shinozuka et al 1994).

DNA condensation for polynucleotide delivery in gene therapy

Polylysine condenses DNA and effects cell transfection (Marquet & Houssier 1991; Behr 1993; Perales et al 1994). However, polylysine has become the subject of more recent interest when covalently attached to a protein, such as transferrin (Cotten et al 1990), as a targeting moiety. This protein then enables target cell penetration by receptor-mediated endocytosis. However, in order to avoid enzymatic lysosomal degradation, the inclusion of free chloroquine (Cotten et al 1990) or the attachment of replication-deficient adenoviruses (Wagner et al 1992) is required to ensure better survival and more efficient transfer of the foreign DNA into the cytosol of the target cell. Other polyamine based gene-delivery systems include polyethylenimine, an organic macromolecule with a high cationic charge density potential which should facilitate DNA condensation and pH buffering (Boussif et al 1995). These applications of polyamines mimic the natural effects of histones interacting with DNA (Ong et al 1976). Starburst polyamidoamine dendrimers are a new class of highly branched spherical polymers that are soluble in aqueous solution and have a unique surface of primary amino functional groups (Tomalia et al 1990; Wu et al 1994; Kukowska-Latallo et al 1996). At physiological pH, these amino groups will be positively charged and should interact with polyanions (phosphates along nucleic acid polymers). Recent studies have shown that certain polyamidoamine dendrimers form stable complexes (aggregates) with DNA under most physiological conditions, some of which are capable of mediating non-specific in-vitro transfection. However, these starburst polyamine dendrimers may not condense DNA into toroidal particles without the use of an additional polycation (DEAE-dextran) (Kukowska-Latallo et al 1996).

The binding of polyamines has a profound effect on DNA structure, causing transitions from B to both A and Z forms of DNA. At higher concentrations, polyamines mediate conformational changes such as DNA aggregation and condensation. Condensation is caused by alleviation of the charge repulsion between neighbouring phosphates on the DNA helix allowing collapse into a more compact structure. DNA condensation is dependent upon three characteristic properties of the natural or synthetic polyamines—the number of positive charges which therefore influence the local ionic strength (Stewart & Gray 1992), the regiochemical distribution of these charges whose pKa's are intimately dependent upon their co-operativity, and the local salt concentration. The prerequisites for delivery of DNA across an intact cytoplasmic membrane are condensation and masking of the negative charges of the phosphate backbone. Spermine (**1**) and spermidine (**2**) are two of the smallest natural polycations capable of effecting both charge neutralization and condensation of the polynucleic acid. These interactions are, however, readily reversible under physiological conditions (Behr 1993). Indeed, many studies (Allison et al 1981; Feuerstein et al 1990; Plum et al 1990; Rowatt & Williams 1992; Stewart & Gray 1992) have shown structure–activity relationships for the binding and condensation of DNA with polyamines, indicating that appropriately modified polyamines are ideally suited for use as gene delivery systems. In order to reinforce these effects, it is apparently beneficial if a lipid is covalently bound to the polyamine (Behr 1986).

At present, most gene therapy protocols involve the use of highly efficient recombinant viral vectors. These gene vectors, however, have a limited carrier capacity and are associated with immuno-

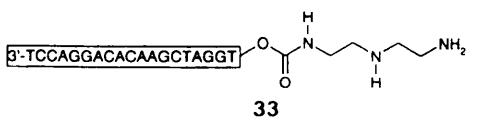

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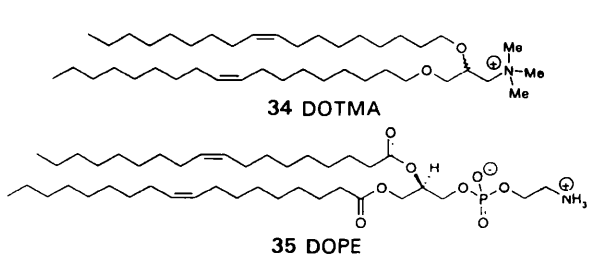
Figure 11. DNA–ethylenediamine conjugate (**33**) as a synthetic RNase.



34 DOTMA

35 DOPE

Figure 12. Structures of Lipofectin components DOTMA (**34**) and DOPE (**35**).

logical problems as a function of high dose or repeated use (Temin 1990). Synthetic vectors could, in principle, solve these problems, and the design of such systems has recently become an area of considerable research interest. Lipofectin (Felgner et al 1987) (Figure 12), was the first cationic lipid formulation to receive widespread attention as a gene delivery agent. Lipofectin consists of a 1:1 mixture of (mono-)cationic lipid *bis*-ether (2,3-dioleyloxy)propyl-*N*,*N*,*N*-trimethylammonium chloride (DOTMA, **34**) and fusogenic lipid diester dioleoylphosphatidylethanolamine (DOPE, **35**, Figure 12). As the cationic lipid requires the presence of a phosphatidylethanolamine (e.g. DOPE) capable of destabilizing bilayer membranes and promoting membrane fusion, it has been postulated (Walker et al 1996) that the encapsulated DNA must gain entry to the cytoplasm by fusion or destabilization of the plasma or endosomal membrane.

Dioctadecylamidoglycylspermine (DOGS, Transfectam, **36**), and dipalmitoylphosphatidyl-ethanolamine spermine (DPPES, **37**) were the first polyamine based lipid (lipopolyamine) gene delivery vectors (Behr et al 1989).These molecules contain spermine (**1**) covalently bound to two hydrophobic chains (Figure 13). When mixed with DNA, these polyamine-incorporating vectors cause condensation and formation of self-organized compact nuclear particles with an excess coat of cationic lipid. These compact particles are not cationic liposomes. The fact that DOGS (**36**) does not require the presence of a fusogenic lipid or a diffusing weak base such as chloroquine (which acts by buffering the acidic lysosomal interior), has two possible explanations—either the particles gain access to the cells via direct membrane fusion, or the DNA escapes from the degradative lysosomal enzymes because of the unique buffering capacity of spermine (Remy et al 1994). This interpretation is supported by an analysis of the pKa's of DOGS (**36**) which are 10·5, 9·5, 8·4 and 5·5. It may indeed be significant that the fourth (lowest) of these pKa values corresponds to the internal pH of the (acidic) lysosome (Remy et al 1994).

Cationic facial amphiphiles (molecules whose hydrophilic and hydrophobic regions are segregated along the long axis) are another polyamine-based system showing promise for gene delivery (Walker et al 1996). Various polyamines—spermine (**1**), tetraethylenepentamine (**38**), and pentaethylenehexamine(**39**)—have been conjugated to bile acid based amphiphiles and then mixed with DOPE (**35**) (1:1) to facilitate transfection. To date, *bis*-glycosylated *cis*-AB-steroid (**40**), a 3α, 7α, 12α-cholic acid amide linked to pentaethylenehexamine (Figure 14) has shown the greatest ability to promote β-galactosidase plasmid uptake in COS-7 cells (Walker et al 1996).

The pKa values of tetraethylenepentamine (**38**) are reported as 10·0, 9·2, 8·2, 4·1 and 2·6 (Paoletti et al 1973). This is an exquisite example of the cooperativity of pKa's along a polymethylene chain, as the fourth pKa is comparable with acetic acid (pKa 4·76), and the fifth with chloro- (pKa 2·87) and fluoroacetic acids (pKa 2·59) (Albert & Serjeant 1984). Therefore, at pH 7·0, a +3 charge for the bile acid monoacylated conjugate of pentaethylenehexamine is assumed (Walker et al 1996). While it is also assumed that the polyamine moiety of this cholic acid conjugate will bind to DNA and cause condensation, the complete

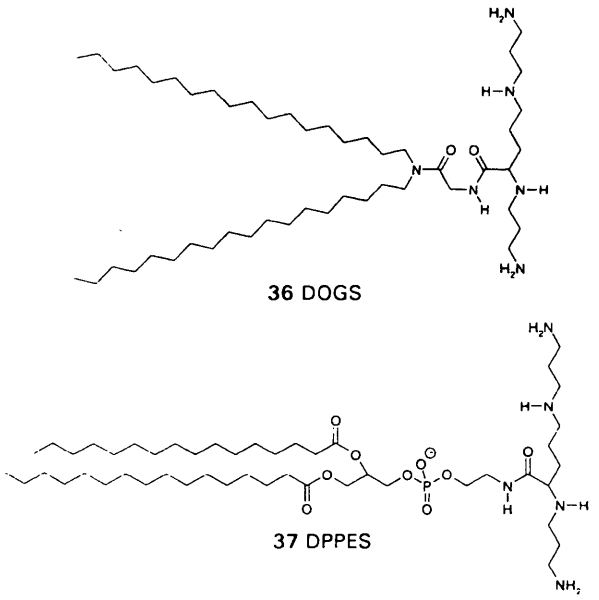


Figure 13. Structures of lipopolyamines DOGS (**36**) and DPPES (**37**).

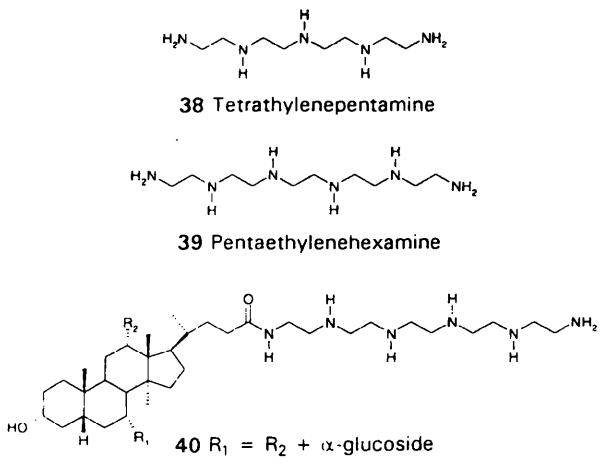


Figure 14. Polyamines and a glycosylated cholic acid conjugate (**40**).

mechanism of DNA uptake, mediated by this synthetic vector, is unclear. One possible theory is that the destabilizing properties of facial amphiphiles (i.e. molecules which possess a nonpolar steroid nucleus with a polar side chain) might increase the fusogenic potential of the transfecting particle.

Spermidine– (**41**) and spermine–cholesterol (**42**) (Figure 15), with spermidine carbamoylated at N^1 or N^3, and used as an unknown mixture of these two +2 charged regioisomers, are novel transfection agents (Guy-Caffey et al 1995). The mechanism by which these compounds promote DNA delivery is unknown, but it is possible that the cationic portion interacts with the nucleic acid, while the hydrophobic cholesteryl moiety associates with the membrane lipid bilayer, resulting in fusion with, or transient disruption of the cell membrane effecting direct delivery of DNA to the cytosol (Guy-Caffey et al 1995). Another cationic cholesterol transfection agent is cholesteryl–spermidine (**43**) (alkylated at N^2, therefore potentially a +3 charged species) (Moradpour et al 1996) (Figure 15), similar to DOGS (**36**) and (like DOGS) not dependent on the presence of a fusogenic lipid for DNA delivery to the cell. A detailed analysis of the structure and formulation of cationic lipids which are efficient in achieving gene delivery to the lung, with particular respect to cystic fibrosis, has established that cationic lipids can be as effective as adenovirus-based vectors (Lee et al 1996). Possibly more significant is the conclusion that the activity of cationic lipids for in-vivo gene delivery could not be predicted from the in-vitro analysis, and therefore this has to be tested directly (Lee et al 1996).

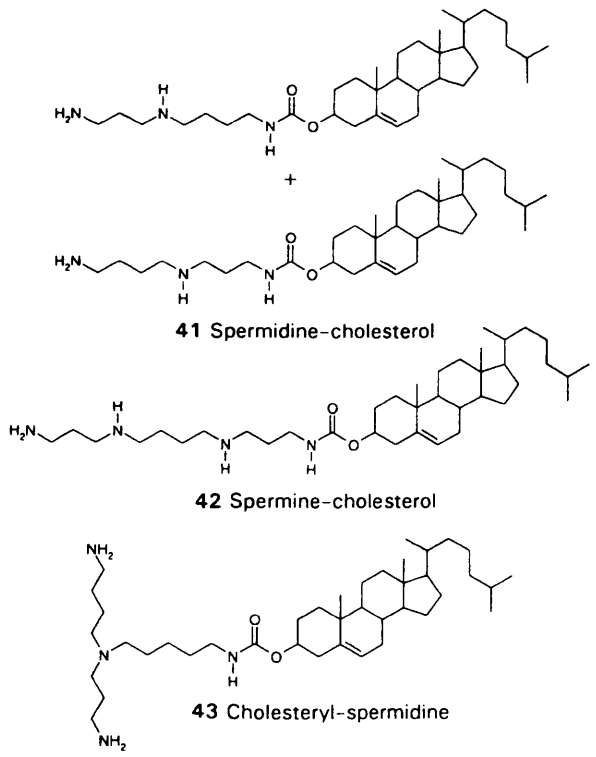


Figure 15. Structures of polyamine-containing cholesterol transfection agents.

Conclusions

Polyamines and polyamine amides continue to demonstrate significant potential in the pharmaceutical sciences both as lead compounds for a variety of therapeutic targets, and as synthetic vectors in gene therapy for efficiently effecting gene delivery.

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Optimization of the MTT Assay for B16 Murine Melanoma Cells and its Application in Assessing Growth Inhibition by Polyamines and Novel Polyamine Conjugates

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Abstract

As part of our continuing development of new cytotoxins with potential anticancer activity, we have synthesized polyamine conjugates containing both the linear tetra-amine spermine and an acridine unit.

Studies of growth inhibition by these novel conjugates and spermine were carried out using B16 murine melanoma cells. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) bioassay has been optimized for this cell line with respect to incubation time for MTT metabolism, inoculum density and incubation period.

A linear relationship has been established between the amount of formazan produced and the number of viable B16 murine melanoma cells. Furthermore, the optimal incubation time of cells with MTT is 3 h. Beyond this time no further significant amount of formazan was generated. The optimal seeding density was determined (4000 cells/well for 2- or 3-day experiments), and we estimate that the doubling time for B16 cells is approximately 24 h. In 48-h assays, spermine (**1**) inhibits cell growth with an EC50 of 450 μM, acylated analogue (**5**) has an EC50 of 5 μM, almost a hundred-fold increase in potency over spermine, and the aniline analogue (**6**) has an EC50 of 1 μM, a five-fold increase in potency over amide analogue (**5**). The EC50 for spermine (**1**) and aniline (**6**) changed little over 3 and 6 days compared with the EC50 over 2 days.

We conclude that there is little significant metabolic influence (with time) on the observed toxicity data. We have also shown that the synthetic conjugates combining both an intercalator and groove binder show greater cytotoxicity than compounds that exhibit just one of these modes of binding. These analogues therefore offer a new lead in the design of cytotoxic polyamines with potential anticancer activity.

Many biological assays require the measurement of surviving and proliferating cells. This can be achieved by several methods, including counting cells that include or exclude dye as an indicator of cell membrane integrity (Hoskins et al 1956), chromium release in which radioactive chromate bound to cellular protein is released as a function of cell lysis (Fass & Fefer 1972), measuring incorporation of radioactive DNA precursors such as [^{3}H]thymidine or [^{125}I]iodo-deoxyuridine during cell proliferation as an index correlating inhibition of DNA synthesis with cell death (Brereton et al 1975), and measuring the metabolism of tetrazolium bromide by the dehydrogenase enzymes in the mitochondria of living cells, as in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) bioassay (Mosmann 1983).

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As part of our continuing research into novel polyamine-based compounds with anticancer activity, we have synthesized small molecules based on the naturally occurring polyamine spermine (**1**, Figure 1), so that these synthetic analogues will bind to DNA with potential cytotoxic results. Polyamines are structurally simple aliphatic compounds consisting of two or three flexible carbon chains connected by basic nitrogen atoms that are protonated at physiological pH (Takeda et al 1983; Usherwood & Blagbrough 1989). They are recognized as playing an important role in many cellular processes including cell growth and replication (Heby & Persson 1990). Polyamines have also

demonstrated anticancer activity which is attributed to DNA binding and interference with DNA transcription (Feuerstein et al 1990), general depletion of polyamine pools and down-regulation of enzymes such as ornithine decarboxylase and spermine–spermidine-N^1-acetyltransferase (Porter et al 1991). Notable examples of these synthetic polyamines are diethylnorspermine (DENSPM) (**2**), diethylspermine (DESPM) (**3**) and diethylhomospermine (DEHSPM) (**4**) (Bergeron et al 1989, Figure 1). Our novel analogues are composed of a polyamine conjugated to the 9 position of acridine, either through an amide bond (**5**) or directly as the aniline (**6**) (Figure 1). Polyaromatics such as acridine are known to bind to DNA through intercalation (Wilson 1996 and acridine analogues have been shown to have anticancer activity (Denny et al 1983). We anticipated that our conjugates will show bifunctional modes of DNA binding (Adlam et al 1994; Rodger et al 1994, 1995) and hence enhanced cytotoxicity over either the polyamine or polyaromatic moiety alone (Carrington et al 1996).

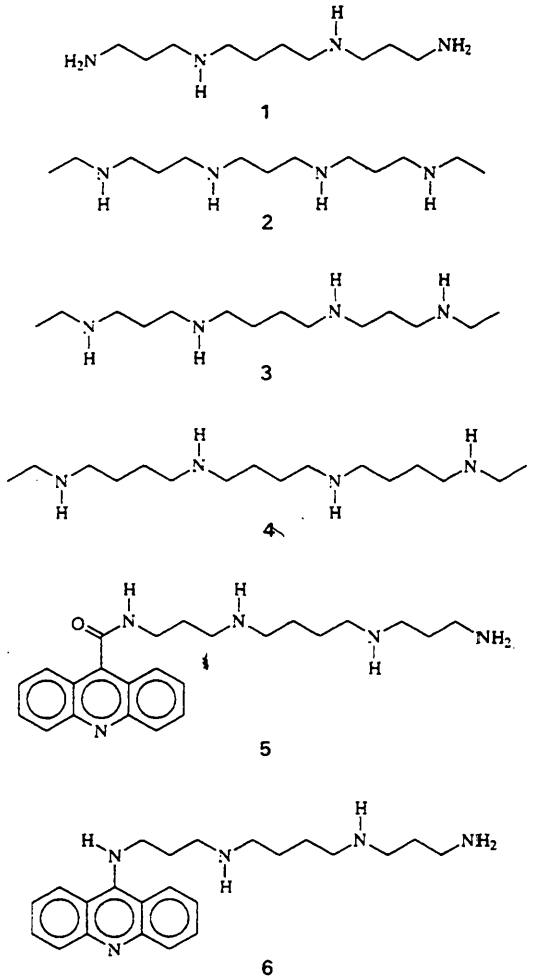

Figure 1. Structures of spermine (**1**), diethylnorspermine (**2**), diethylspermine (**3**), diethylhomospermine (**4**) and the two synthetic spermine conjugates amide (**5**) and aniline (**6**).

To make an initial assessment of the growth inhibitory activity of our synthetic conjugates, we carried out in-vitro studies using B16 murine melanoma cells to obtain EC50 values. We chose the MTT assay with continuous drug exposure as it involves no washing or cell transference steps and leads to rapid, sensitive and reliable indices of growth. Furthermore, there is reproducible drug sensitivity in an individual cell line over the course of multiple passages and over several months of cultivation (Alley et al 1988).

In this assay, the tetrazolium ring in MTT is cleaved by dehydrogenase enzymes in the active mitochondria of living cells, converting the water-soluble yellow tetrazolium salt to the insoluble blue formazan crystals. Dissolving these crystals in dimethylsulphoxide allows determination of the number of viable cells present at the time of MTT addition by colorimetric assessment. This approach to in-vitro testing has been successfully used for large scale screening of potential new anti-neoplastic drugs by the National Cancer Institute in the USA.

As a literature search failed to reveal any record of the MTT assay being applied to cultures of the B16 murine melanoma cell line, it was necessary to optimize the inoculum density and incubation period to estimate the cell growth rate and ensure that drug assays were conducted in the logarithmic phase of cell growth. It was also necessary to determine the optimum time for the viable cells to metabolize the available MTT, and to prove that the relationship between cell number and absorbance of the formazan metabolite is linear. We address these issues as part of our optimization of the MTT assay for B16 murine melanoma cells, and apply this assay to assess the growth inhibition of murine melanoma cells by polyamines and by novel polyamine conjugates.

Materials and methods

Chemistry

The synthetic conjugates were prepared by acylation or alkylation of acridine species with spermine bearing three amine protecting groups, leaving one nucleophilic primary amine functional group free to react. Acridine-9-carbonyl-N^1-spermine was prepared as previously reported (Carrington et al 1996) from acridine-9-carboxylic acid and N^1, N^2, N^3-tribenzyloxycarbonyl-spermine. Acridine-9-(N^1-spermine) was prepared by first reacting 9(10H)-acridone with thionyl chloride (DMF 2 drops, heated under reflux, 0·5 h) to yield 9-chlor-

oacridine (96%). This was then stirred with solid NaOH in molten phenol (18 h) to afford 9-phenoxyacridine (99%). Displacement of phenoxide by N^1,N^2,N^3-tribenzyloxycarbonyl-spermine was achieved by stirring the reactants in molten phenol (18 h) to yield, after silica gel chromatography, the protected intermediate (44%). The target compound was obtained in 90% yield after catalytic hydrogenolysis over 10% palladium on carbon.

Cell culture

B16 murine melanoma cells were cultured in RPMI 1640 media (Gibco) containing 10% heat-inactivated foetal bovine serum (ICN), 2 mM L-glutamine, 1% non-essential amino acids, 50 int. units mL^{-1} penicillin, and 50 μg mL^{-1} streptomycin in a humidified atmosphere of 95% air and 5% CO_2.

MTT assay

All MTT assays were carried out in 8 × 12-well culture plates (Nunc) using a standard configuration of wells containing B16 murine melanoma cells in RPMI media containing a test compound, cells in media, or test compound in media. The wells lacking drug served as a control for cell growth. The wells without cells were blank controls during spectrophotometric detection of MTT, allowing for chemical reduction of MTT, and for contributions to absorbance by chromogenic drug solutions. The 96-well plates were incubated at 37°C for the required time periods, washed with serum-free RPMI 1640 media, and then incubated with 1 mg mL^{-1} MTT in serum-free RPMI media for the predetermined optimal period. The blue formazan crystals produced by viable cells were dissolved in dimethylsulphoxide to allow assay using a microplate spectrophotometer (Perkin-Elmer) at 540 nm (test) and 690 nm (background). The measured absorbances were used to estimate EC50 values for the test compounds.

Results and Discussion

Before using the MTT assay to assess the cytotoxicity of antineoplastic agents on B16 cells, control experiments were performed to ascertain the relationship between B16 cell number and the amount of tetrazolium metabolized to formazan. The optimal time required for the metabolism of tetrazolium was also determined (Figure 2). This confirms the linear relationship between the amount of formazan produced and the number of viable B16 murine melanoma cells. It also shows the optimal incubation time of cells with MTT to be 3 h, as beyond this time no further significant amount of formazan was generated.

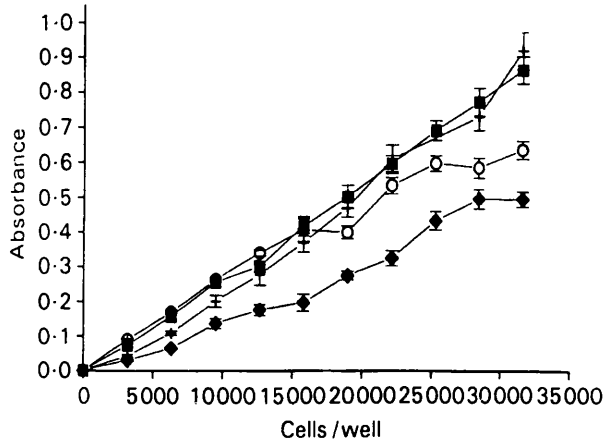

Figure 2. Correlation of viable B16 murine melanoma cell number against the absorbance of metabolized MTT (at 540 nm test wavelength less 690 nm background) after incubation of cells with MTT for ◆ 1 h; ○ 2 h; + 3 h and ■ 4 h. Each point represents the mean value for eight independent determinations performed in one experiment.

The optimal seeding density was determined over several time periods (Figure 3) allowing us to estimate the doubling time for B16 cells to be approximately 24 h. From this we determined that for experiments of 48 or 72-h duration the optimal seeding density is 4000 cells/well. For 6-day experiments, the optimal density was 1000 cells/well (data not shown). These initial seeding densities gave enough cell growth to allow conveniently large amounts of formazan metabolite to be generated on addition of MTT, without limiting the growth of the cells as they become confluent.

The results from the assays over 48 h are shown in Figure 4. Spermine (1) inhibits cell growth with an

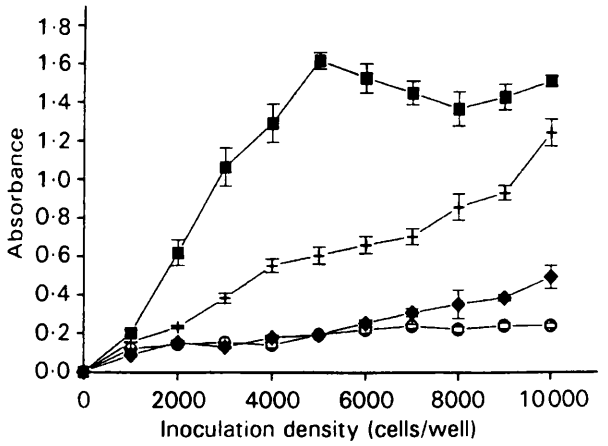

Figure 3. Correlation of absorbance of metabolized MTT by B16 cells with their inoculation density after incubation in RPMI media for ○ 0 h; ◆ 24 h; + 48 h or ■ 72 h. Each point represents the mean value for eight independent determinations performed in one experiment.

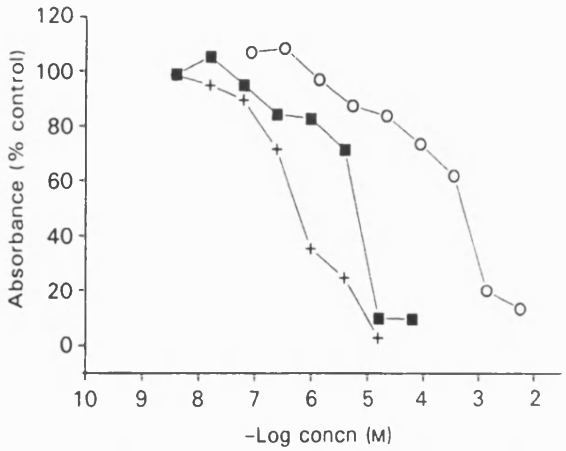

Figure 4. Dose-dependent growth inhibitory effect of ○ spermine (**1**) and synthetic conjugates ■ **5** and + **6**, following incubation with B16 cells for 48 h as estimated by the MTT assay. Each curve represents the average of three independent assays with six replicate samples for each data point. Error bars have been omitted for clarity (s.e. of all values < 20%).

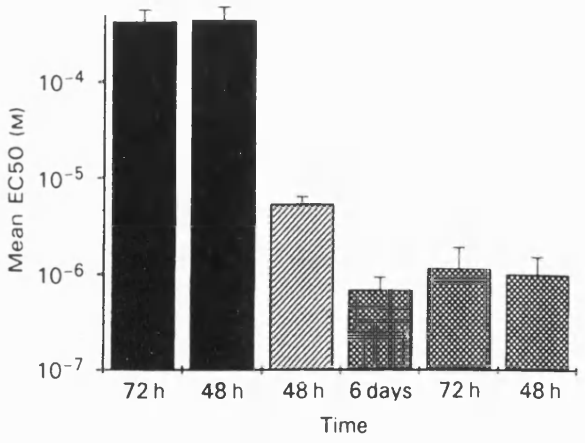

Figure 6. Mean EC50 values for ■ spermine (**1**) and synthetic conjugates ▨ **5** and ▩ **6** as determined using the MTT assay after incubation with B16 murine melanoma cells for various time periods. Each value represents the average of three independent assays with six replicate samples for each data point.

EC50 of 450 μM, the acylated analogue (**5**) has an EC50 of 5 μM, almost a hundred-fold increase in potency over spermine, and the alkylated (aniline) analogue (**6**) has an EC50 of 1 μM, approximately a five-fold increase in potency over amide analogue (**5**). The EC50 for spermine (**1**) and aniline (**6**) changed little over 72 h and 6 days compared with the EC50 over 48 h (Figures 4, 5). Spermine (**1**) was incubated for 48 h and 72 h (Figure 6). Synthetic conjugates (**5**) and (**6**) were tested for 48 h, and up to 6 days with continuous drug exposure throughout these periods. This ensured that minimal growth inhibitory activity due to limited solubility in culture medium and modes of action requiring extended contact with cells were detected (Figure 6).

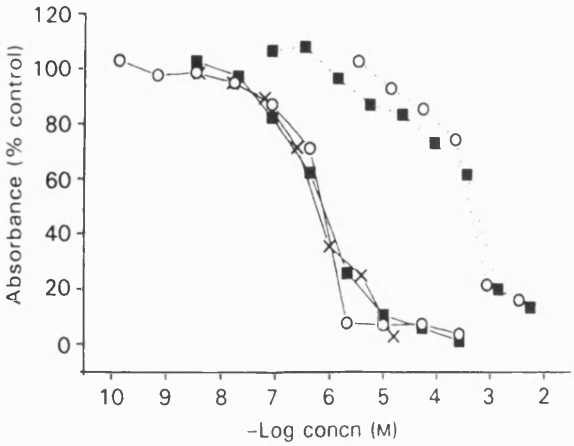

Figure 5. Dose-dependent growth inhibitory effect of – – – spermine (**1**) and —— synthetic conjugate **6** following exposure to B16 cells for ■ 48 h; ○ 72 h or + 6 days as estimated by the MTT assay. Each curve represents the average of three independent assays with six replicate samples for each data point. Error bars have been omitted for clarity (s.e. of all values < 20%).

A comparison of the antiproliferative activity of DENSPM (**2–3**) and DEHSPM (**4**) against L1210 cells in culture, showed average 48 h EC50 values of > 100 μM, 36 μM and 0·4 μM and 96-h EC50 values of 1·3 μM, 0·2 μM and 0·06 μM, respectively, (Bergeron et al 1989). The reason for this increase in cytotoxicity with time was attributed to interference with enzymes in polyamine biosynthetic pathways and hence depletion of polyamine pools. From the results obtained with our conjugates (Figure 6), we conclude that there is little significant metabolic influence (with time) on the observed toxicity data. We have also shown that the synthetic conjugates combining both an intercalator and groove binder show greater cytotoxicity than compounds that exhibit just one of these modes of binding (Carrington et al 1996). These are therefore analogues that offer a new lead in the design of cytotoxic polyamines with potential anticancer activity.

Acknowledgements

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Macrocyclic polyamine lactam synthesis by diphenyl ether closure of 23-, 24- and 28-membered rings

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Novel 23-, 24- and 28-membered cyclic polyamine amides (cinnamamides) have been prepared by closure of diphenyl ethers; functionalized conjugates of spermidine and spermine underwent intramolecular aromatic nucleophilic substitution to afford nitro-substituted analogues of cadabicine class (24-membered polyamine lactam) alkaloids.

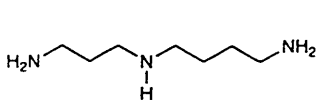
Polyamines such as triamine spermidine **1** and tetraamine spermine **2** are widely distributed in nature and display a variety of biological activities.[1] Cinnamic acid (3-phenylpropenoic acid) conjugates are commonly isolated as the corresponding *N*-substituted amides from plant sources.[2] Ferulic acid (4-hydroxy-3-methoxycinnamic acid) **3** is found as feruloylputrescine, a conjugate of 1,4-diaminobutane.[3] N^1,N^3-Di-(*E*)-feruloylspermidine **4** has been isolated from *Corylus avellana* L.[3,4] Spermidine conjugates have also been found asymmetrically substituted with both ferulic and caffeic (3,4-dihydroxycinnamic) acids **5**.[3] Maytenine,[5] from *Maytenus chuchuhuasha*, is the unsubstituted dicinnamamide of spermidine **1**. Dicinnamamides of spermine **2** include kukoamine A, a biologically active bis(dihydrocaffeoyl) conjugate.[6,7]

Cyclic polyamine amide containing cinnamamides are less common natural products whose biological activities are largely unknown.[2,8] Spermine **2** containing macrocyclic polyamine lactams include *inter alia* chaenorhine and ephedradine A.[8] Spermidine **1** containing polyamine lactams include codonocarpine **6**, from *Codonocarpus australis*,[9] and capparisinine **7**.[10] Cadabicine **8** is a diphenyl ether 24-membered ring containing spermidine **1**, from *Cadaba farinosa* Forsk. The regiochemical substitution of the diphenyl ether moiety is reversed with respect to the unsymmetrical spermidine moiety in **6** and **7**.[11] We are unaware of a synthesis[2,8] of a cadabicine **8** class alkaloid, although a regiocontrolled synthesis of the *Lunaria* diphenyl ether alkaloid codonocarpine **6** has been reported.[12]

Macrocycles containing diphenyl ethers are of chemical and biological interest as they occur in vancomycin and ristocetin antibiotic families.[13–15] Also, these substitution patterns are found in anti-cancer peptide conjugate RA-VII and ACE inhibitor K-13.[15,16] Macrocyclic polyamine lactams incorporating a diphenyl ether have been prepared using intermolecular Ullmann diaryl ether synthesis followed by lactam formation.[8,12] Herein we report the first design and synthesis of 23-, 24- and 28-membered ring polyamine lactam conjugates that are nitro-substituted analogues of cadabicine **8** class alkaloids.

The required substituted cinnamic acids were prepared from the corresponding benzaldehydes by Knoevenagel condensation.[17,18] Isoferulic acid (3-hydroxy-4-methoxycinnamic acid) **9** was prepared in good yield (92%) under standard conditions starting with malonic acid (EtOH, piperidine, pyridine, 3 h, reflux).[17] 4-Fluorobenzaldehyde was similarly converted into 4-fluorocinnamic acid **10** (85%). Nitration *ortho* to fluorine, in order to activate the final intramolecular nucleophilic substitution (S_NiAr) reaction, achieved with conc. nitric acid, afforded 4-fluoro-3-nitrocinnamic acid **11** (1 h, 0 °C, 72%).

For the 23-membered ring **12**, *N*-(3-aminopropyl)-1,3-diaminopropane was protected with trifluoroacetyl groups on the



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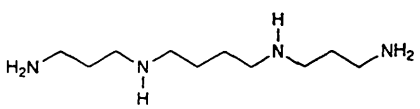

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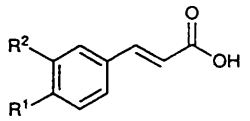
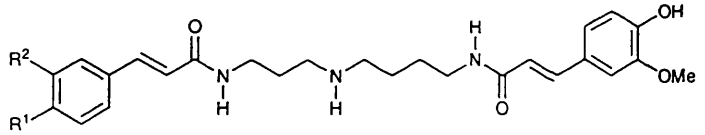
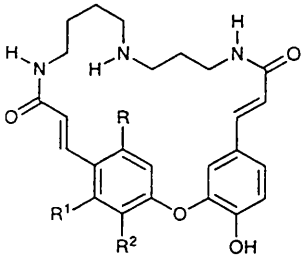


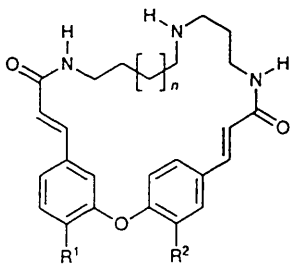
3 R^1 = OH, R^2 = OMe
9 R^1 = OMe, R^2 = OH
10 R^1 = F, R^2 = H
11 R^1 = F, R^2 = NO_2



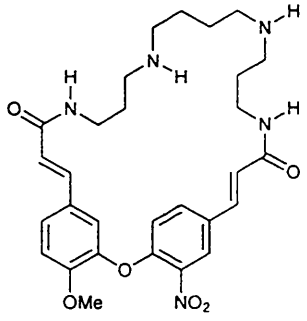
4 R^1 = OH, R^2 = OMe
5 R^1 = OH, R^2 = OH



6 R^1 = H, R^2 = OMe
7 R^1 = OMe, R^2 = H



8 n = 1, R^1 = OH, R^2 = H
12 n = 0, R^1 = OMe, R^2 = NO_2
13 n = 1, R^1 = OMe, R^2 = NO_2
14 n = 1, R^1 = OH, R^2 = NO_2



15

primary amino groups (2 equiv. CF_3CO_2Et, THF, 10 min, 25 °C).[19–21] This was followed by immediate Boc protection of the central, secondary amine (Boc_2O, THF, 18 h, 25 °C). Conc. aq. ammonia was added to the solution of tri-protected triamine until the pH was greater than 11 to remove the trifluoroacetyl protecting groups (24 h). Mono-Boc protected amine was isolated and purified by flash column chromatography (15 : 5 : 1, CH_2Cl_2–MeOH–conc. aq. NH_3, v/v/v, R_f 0.13). The cinnamic acid moieties[17,18] were coupled sequentially (first the isoferuloyl **9** then the 4-fluoro-3-nitro **11**) to the primary amines by pre-activation with 2-mercaptothiazoline (2-thiazoline-2-thiol, thiazolidine-2-thione)[5] (DCC, 0.01 equiv. DMAP, CH_2Cl_2, 1 h, 25 °C, followed by filtration to remove the urea). Mono-Boc protected triamine was added to the yellow CH_2Cl_2 solution of the *N*-acylated 2-mercaptothiazoline and the coupling was typically complete after 3 h (*ca.* 50% each acylation). Cyclisation was brought about by stirring with 5 equiv. CsF in anhydrous DMF (18 h) to give 23-membered ring polyamine lactam **12** (79% isolated yield). *O*-Arylation has occurred by intramolecular aromatic nucleophilic substitution (S_NiAr) reaction of *o*-nitro-activated fluoride by the remote phenol.

For the 24-membered ring **14**, spermidine **1** was reacted with formalin to give a hexahydropyrimidine adduct (0.95 equiv. 37% w/w aq. formaldehyde, H_2O, 1 h, 91%) as developed independently by Ganem and Hesse and their co-workers.[22,23] Isoferulic acid **9**[17,18] was coupled to the primary amine of this regioselectively protected spermidine through the 2-mercaptothiazoline activated intermediate (−78 to 25 °C, 55%). After chromatography, this hexahydropyrimidine was deprotected by heating with malonic acid and pyridine (EtOH, reflux, 2 h, 79%).[22] 4-Fluoro-3-nitrocinnamic acid **11** was coupled to the uncovered primary amine and then the secondary amine was protected by a Boc group (1.1 equiv. Boc_2O, MeOH, 18 h, 25 °C, 89%) to afford a linear precursor of cadabicine analogue **13**. Cyclisation was carried out by stirring with 3 equiv. CsF in anhydrous DMF (18 h, 71% isolated yield), final purification by RP-HPLC (5 μm C8 inertpak column eluting with 1:4 aq. TFA (0.1%)–MeOH, v/v, λ = 250 nm). TFA catalysed deprotection (1:1 TFA–CH_2Cl_2, v/v, 45 min, 0 °C, 90%) of the Boc group in diaryl ether **15** was followed by *O*-demethylation with BBr_3 (1.2 equiv., CH_2Cl_2, 3 h, −78 °C) to give 2′-nitrocadabicine **14** in 60% isolated yield.

For the 28-membered ring **15**, spermine **2** was protected in a similar fashion to *N*-(3-aminopropyl)-1,3-diaminopropane *vide supra*. Trifluoroacetyl groups were used to block the two primary amines then two Boc groups were introduced at the secondary amines. Conc. aq. ammonia was used to remove the trifluoroacetyl protecting groups and the N^2,N^3-diBoc spermine was then purified by chromatography. The two cinnamic acid moieties[17,18] were introduced in a stepwise fashion using 2-mercaptothiazoline activation to yield the cyclisation precursor. Cyclisation was carried out in anhydrous DMSO with 3 equiv. K_2CO_3 and 10 equiv. 18-crown-6, the oxygen nucleophilicity was found to be too low without the crown ether. The cyclisation reaction did not proceed to completion at 25 °C (starting material still present after 24 h). However, heating the mixture to 50 °C, in the presence of 18-crown-6, led to complete reaction after 5 h, yielding the desired macrocycle **15** (66%).

The previously proposed mechanism of cyclisation involved bringing the two sites of reaction into proximity by π-orbital stacking interactions between the electron rich guaiacol (2-methoxyphenol) ring and the electron deficient *o*-fluoronitrophenyl ring.[15] However, macrocycle formations of this type have recently been demonstrated to proceed in good yield when the aryl hydroxy group is replaced by an alkyl hydroxy group, proving that such π–π interactions are not necessary for successful cyclisation.[24] As the isolated yields are high, this practical approach by aromatic nucleophilic substitution (intramolecular S_NiAr reaction) should find ready application in the synthesis of natural products and their analogues with particular reference to cyclic spermidine and spermine alkaloids of the codonocarpine **6** and cadabicine **8** classes.

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